ERRATUM

"Degeneration of cross striated musculature in Vitamin E-low rats", by H. M. Evans, G. A. Emerson and I. R. Telford, 1938, **38**, 625, line 15 should read, "Olcott who has reported microscopic changes strikingly . . . ."

Proceedings

of the

Society

for

Experimental Biology and Medicine

VOL. 39. NOVEMBER, 1938. No. 2.

SECTION MEETINGS

CLEVELAND		
Institute of Pathology	October	14, 1938
MISSOURI		
St. Louis University Medical School	October	12, 1938
NEW YORK		
College of Physicians and Surgeons	November	23, 1938
PACIFIC COAST		
Stanford University	October	15, 1938
SOUTHERN CALIFORNIA		
University of Southern California	October	25, 1938
WESTERN NEW YORK		
Cornell University	October	29, 1938

10155

Preparation and Properties of the Charcoal Adsorbate of Liver Extract.

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From the Thomas Henry Simpson Memorial Institute for Medical Research, University of Michigan.

A method for the preparation of a concentrated liver extract was described based on the precipitation of protein with lead acetate and the adsorption of the active principle by charcoal.[1] The use of a lead salt had some disadvantages, so a method has been developed which eliminates the use of this substance.

[1] Kyer, J. L., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1102.

The present method has been employed for 2 years and the extract has been found to be potent both in inducing remissions in patients with pernicious anemia in relapse and in the maintenance of normal red blood cell levels.

Five pounds of fresh liver are ground and suspended in 5 liters of water. The mixture is heated in a water bath to 80°C and filtered. The residue is washed with hot water and the combined filtrates are concentrated to about one liter. Ethyl alcohol, 95%, is then added to a final concentration of 70% and the mixture is filtered. The alcohol is removed from the filtrate by distillation *in vacuo* and to the water extract, after filtration, is added a solution of 27.5 g of anhydrous $CaCl_2$ followed by the addition of 26.5 g of anhydrous Na_2CO_3. The formation of the $CaCO_3$ brings down a considerable amount of material and appears to accomplish the same result as the use of lead acetate in our previous method. After filtration the clear yellow solution is adjusted to pH 5 with hydrochloric acid and 25 g of norit charcoal are added. The mixture is shaken frequently over a period of an hour and the norit is filtered off by suction. After washing with a small amount of water, 10 g of norit are added to the filtrate and the mixture is shaken for a short time. After filtration the norit residues are eluted with 400 cc of hot 50% alcohol. The alcoholic extract is filtered off by suction, and the elution in hot alcohol is repeated. The combined alcoholic extracts are concentrated *in vacuo* until 3 cc represent 100 g of fresh liver. This light yellow solution is allowed to stand over night in the ice box and is then filtered, bottled, and sterilized.

The data in Table I show the reticulocyte response in a patient with pernicious anemia in relapse, receiving one 3 cc injection of the extract.

Normal red blood cell levels have been maintained in several patients by treatment with the concentrated extract for varying

TABLE I.

Days after Treatment	Red Blood Cells (millions per mm^3)	Reticulocytes %
0	1.53	2.0
1		1.5
2		5.9
3		15.4
4		32.5
5		39.6
6		44.4
7	1.87	20.3
8		21.1
9		9.9
10		5.3

periods up to 2 years. One patient has received a 3 cc injection on an average of once every 15 days for 9 months. Two patients have had one 3 cc injection every 2 weeks, one for over a year and the other for about 6 months. It has been found necessary to administer 3 cc weekly to 2 patients to maintain a normal red blood cell level.

In the investigation of some of the properties of the extract determinations of total solids and total nitrogen were made on 12 individual lots derived from 5 pounds of fresh liver and one lot derived from 10 pounds. A marked difference may occur between individual lots of liver without apparent variation in potency and the total solid content has varied from 3.9% to 14.6% while the total nitrogen values have ranged from 0.41% to 0.91%. The average of the individual determinations gave a total solid content of 6.3% or 189 mg per 3 cc, and a total nitrogen of 0.62% or 18.6 mg per 3 cc.

The extract reduces alkaline copper solution, is precipitated by phosphotungstic acid, and gives a positive biuret reaction. Saturation with ammonium sulfate produces a precipitate which is active in inducing a reticulocyte response but is not so potent as the original solution.

The material filtered off after concentration of the alcoholic elute and cooling in the ice box gives a positive murexide test and is difficultly soluble in cold water. This substance resembles the complex purine described by Subbarow, Jacobson and Fiske[2] and classified by Jacobson and Subbarow[3] as an "accessory" factor which augments the action of a primary factor. This material has always been discarded in our preparations since the concentrate from which it is filtered has shown satisfactory hematopoietic activity.

Tyrosine, which has also been called one of the accessory factors,[3] has been found in charcoal filtrates of liver extract by Subbarow, Jacobson and Fiske.[2] Using both the Millon reaction and Folin-Marenzi procedure, tyrosine was found in our final product which is a charcoal adsorbate, not a filtrate. The charcoal filtrates have also been tested and no tyrosine could be detected. This is in disagreement with the original work of the above authors[2] but may be due to the use of a particular type of charcoal. We have observed that 0.5 g of our norit adsorbs all the tyrosine from 10 cc (10 mg tyrosine) of the standard tyrosine solution used in the Folin-

2 Subbarow, Y., Jacobson, B. M., and Fiske, C. H., *New Eng. J. Med.*, 1935, **212**, 663.

3 Jacobson, B. M., and Subbarow, Y., *J. Clin. Invest.*, 1937, **16**, 573.

Marenzi procedure, while with the use of a greater amount of another type of charcoal tyrosine could be detected in the charcoal filtrate.

By use of the method of preparation here described the concentrated liver extract contains tyrosine, which Subbarow, Jacobson and Fiske[2] postulate as one of the factors necessary for blood formation.

Summary. 1. A method for the preparation of a concentrated liver extract is described in which the precipitation of $CaCO_3$ by the interaction of $CaCl_2$ and Na_2CO_3 is used to remove protein, and the active principle is subsequently adsorbed on norit. Of this extract 3 cc are derived from 100 g of fresh liver. 2. The extract is effective in the treatment of pernicious anemia in relapse and in the maintenance of a normal red blood cell level. 3. Some properties of the extract are discussed. 4. Tyrosine is present in the final product.

10156 P

Determination of "Hormone Iodine" in 5 cc. Blood.*

J. F. MCCLENDON AND WILLIAM FOSTER.

From the Laboratory of Physiological Chemistry, University of Minnesota, Minneapolis.

In a series of 100 determinations of blood iodine by the McClendon-Bratton method it appeared as if a long time was required to eliminate a dose of Lugol's solution from the blood.

This suggested the desirability of developing a method for fractionating quantitatively the iodine of blood. We found when a sample of blood is divided into 2 parts and to 1 part KI is added and then the blood samples are subjected to the following procedure that the KI does not appreciably raise the blood iodine.

Five cc of blood is spurted through a fine opening into 100 cc of methanol in a glass-stoppered flask of capacity of about 115 cc to the stopper. This is violently shaken and then allowed to settle. The methanol is decanted and 100 cc of acetone introduced and the shaking repeated. An 8-inch length of 3/8-inch Visking sausage casing is closed at one end by any method, and the other end tied onto a 100 cc burette without stop-cock. The acetone suspension is shaken and poured into the burette. The acetone filters through

* Aided by grants from the University of Minnesota and the Therapeutic Research Committee, American Medical Association.

the casing and the blood residue remains in the casing. After the visible liquid has evaporated, the casing is suspended in air and dried 24 hours or in an oven at 100° and dried for one hour and then analyzed by the McClendon-Bratton method.[1]

It was shown that although KI is washed out of the blood by this means that thyroglobulin-iodine added to the blood is entirely retained. Therefore the method is provisionally considered a method of determining "hormone iodine."

	Hormone Iodine
5 cc beef blood	0.55 γ
5 cc beef blood + 0.5 γ thyroglobulin-iodine	1.04 γ
5 cc beef blood + 1 γ iodine as KI	0.56 γ

The values of 125 determinations on human blood obtained by this method are near 0.3 γ in 5 cc whereas the normal of total blood iodine is near 0.5 γ in 5 cc. Taking 2.5 g iodide by mouth did not greatly raise the "hormone iodine."

10157 P

Effect of Solutions of Salts Normally Present in the Body on Imbibition of Water by Brain Tissue *in Vitro*.*

A. E. TAFT. (Introduced by S. Mudd.)

From the Psychiatric Laboratory, Philadelphia General Hospital.

Earlier experiments[1] on the effects of solutions of various salts on brain cells have led indirectly to this study of imbibition of water in salt solutions by whole brains of white rats.

The rat's brain was chosen in order to find the reaction of the whole organ, so that the relation of surface to mass should be relatively constant. As might have been anticipated, Parry[2] found this relation to be a factor in variations in the degree of swelling of muscle tissue.

If the swelling is not allowed to go on until a constant is reached,

1 McClendon, J. F., and Bratton, A. C., *J. Biol. Chem.*, 1938, **123**, 699.

* Aid was received through a grant from a fund at the Gladwyne Research Laboratory, by the courtesy of Dr. Ludlum. Acknowledgment is made to Dr. Reinhold, Biochemist of Philadelphia General Hospital, for supplying the formulæ for the salt solutions.

1 Ludlum, S. DeW., Taft, A. E., and Nugent, R. L., *Arch. Neur. and Psy.*, 1930, **23**, 1121.

2 Parry, A. A., *J. Cell. and Comp. Physiol.*, 1936, **8**, 277.

comparisons are open to error, and since brain tissue is so rapidly hydrated when left in water or in salt solutions of any degree of hypotonicity, it seemed that small total brains might yield more satisfactory results than larger brains or blocks of tissue. In the case of the latter, the difference in the swelling capacities of gray and white matter is a point of special significance in making comparisons.

The brains were removed from young animals up to 10 days of age after decapitation; in older ones, after ether. The removal was done rapidly, they were weighed immediately and placed at once in the various solutions. The results are based on more than 300 weighings.

The fluid series was made up of distilled water alone, Ringer-Tyrode solution, sodium, potassium, magnesium and calcium chlorides in distilled water at a concentration iso-osmotic with Ringer's solution. This was made according to Höber[3]: NaCl 0.8%; $NaHCO_2$ 0.1; KCl 0.02; $CaCl_2$ 0.02; $MgCl_2$ 0.01; NaH_2PO_4 0.005.

After standing 24 hours in the several solutions in the refrigerator, the specimens were weighed again, with particular care in draining and deducting the weight of excess water which collected in the weighing pan during the process of weighing.

There was more or less difference in the individual results, but for the sake of brevity the average of the swelling quotients, expressed in terms of gain in weight, are presented:

H_2O	Ringer	NaCl	KCl	$MgCl_2$	$CaCl_2$
2.56	1.37	1.36	1.48	1.39	1.25

These weights have the same general relations as those in the individual series. The standard error (variation) is ± 0.03, except for KCl, ± 0.05.

A second set of similar experiments was made in which the brains were allowed to stand in the same series of salt solutions for 90 minutes, at room temperature, when they were changed to distilled water for 24 hours in the refrigerator with these results:

H_2O	Ringer	NaCl	KCl	$MgCl_2$	$CaCl_2$
2.56	2.44	2.26	2.16	1.90	1.71

The standard error (variation) in this series is ± 0.1; this value applies to H_2O throughout.

In the first series, in which only salt solutions were used, the difference between the value for water alone and those for the salt

[3] Höber, R., *Physikalische Chemie der Zelle und der Gewebe*, 6th Ed., 1926, p. 666.

solutions is clear, with the figure for Ca notably smaller than the others.

In the second series, in which the salt solutions were followed by distilled water, the difference between the figures for water alone, and after the salt solutions is relatively little, except in the instances of Mg and Ca.

Since the same anion is present in both series, in each case of the individual salts, the bi-valent cations are apparently the chief factors of influence.

Where the same points are concerned, these results correspond in general to those of Haldi[4] and his associates in relation to swelling of brain tissue. They indicate an effect of Ca on brain tissue similar to that reported by Höber[5] on the plasma membrane and other parts of various cells, and to the findings of Langmuir and Blodgett[6] on the effect of salt solutions on mono-layers, and those of Herbst,[7] who observed that the individual cells of developing echinoderm eggs fall apart in Ca-free water, but are united again on the addition of Ca.

The findings have a bearing on the importance of these salts in maintaining normal water relations in the body tissues, notably in the brain.

10158

Vitamin C Nutrition in Artificial Fever.

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A number of studies have shown that Vitamin C requirement is increased in pneumonia, tuberculosis, and other diseases. It has been suggested that the decreased Vitamin C excretion in pneumonia may signify a greater requirement because of an increased metabolic rate.[1] Heise and Martin[2] have shown an increased rate of utiliza-

4 Haldi, J. A., and Rauth, J. W., *Am. J. Physiol.*, 1925-26, **75**, 294; *ibid.*, 1927, **80**, 631.

5 Höber, R., *loc. cit.*, p. 695.

6 Langmuir, I., and Blodgett, K. B., *Koll. Z.*, 1936, **73**, 257.

7 Herbst, C., in Höber, p. 695.

1 Bullowa, J. G. M., Rothstein, I. A., Ratish, H. D., and Harde, E., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 1.

2 Heise, F. H., and Martin, G. J., *Am. J. Dig. Dis. and Nutrit.*, 1937, **4**, 368.

tion or destruction of Vitamin C in tuberculosis. The assumption has been made that fever will increase Vitamin C requirement, but this has not been proved by direct experimental evidence. This paper is the report of an investigation of the effect of artificial fever on Vitamin C stores in guinea pigs and upon Vitamin C excretion in man.

Guinea Pigs: In order to study the rate of depletion of Vitamin C stores, all pigs were fed a scorbutic diet.[3] Twenty-three pigs were used as controls and 21 were given fever treatment. In a warm air chamber heated with light bulbs, temperatures were raised daily to 105°-106° for 2 to 6 hours. Temperatures were easily raised to 106° with a chamber temperature of 100°-102°. Groups of pigs were killed after 4, 7, 15 and 25 days and tissues analyzed by the method of Bessey and King.[4] Adrenals and kidneys of all animals and the brains of a few were analyzed. With the method used, reliable figures could not be obtained on liver extracts.

Results recorded in Table I show that Vitamin C stores are depleted faster in animals with fever than in their controls. However, when a state of scurvy is approached at 15 days, there is little difference between controls and fever treated animals. Since Vitamin C is stored in the adrenals, the rate of Vitamin C depletion in them should be more significant than great depletion in other tissues. When the results are analyzed with the method described by Dunn,[5] a highly significant difference is found between controls and fever treated pigs. There is approximately one chance in 4000 and one in 25,000,000 in the 4- and 7-day periods respectively that these results occur by pure chance.

TABLE I.
Effect of Fever on Vitamin C Stores of Adrenals and Kidneys.

	Fever treated pigs				Controls		
Days on diet	No. of pigs	Total hr of fever	Mg Vit. C per g tissue		No. of pigs	Mg Vit. C per g tissue	
			Adrenals	Kidneys		Adrenals	Kidneys
4	7	13	.17 ± .0126	.044 ± .007	7	.278 ± .041	.048 ± .017
7	7	15	.13 ± .0038	.020 ± .0003	7	.188 ± .016	.028 ± .002
15	4	30	.053	.018	4	.084	.021
25	3	45	.042	.017	5	.046	.018

Vitamin C concentrations found in the kidneys are less significant but interesting. After 4 days there is not a significant difference

[3] Eddy, Walter H., *Am. J. Publ. Health*, 1929, **19**, 1309.

[4] Bessey, Otto A., and King, C. G., *J. Biol. Chem.*, 1933, **103**, 687.

[5] Dunn, Halbert L., *Physiol. Rev.*, 1929, **9**, 275.

between the 2 groups. After 7 days there is a difference which is as significant as values found in the adrenals for the corresponding period. Seven days, with 15 hours of fever, lowered the concentration to .02 mg per g of tissue which corresponds to 15 days for the controls. This is apparently the scorbutic level because only slightly lower values were obtained in pigs with severe scurvy.

A few analyses were made on the brains which indicated only slight loss of Vitamin C. One animal which had .07 mg per g of tissue in the adrenals still had .12 mg per g of tissue in the brain compared with a normal value of .14 to .16 mg per g.

Scurvy appeared in two fever treated pigs 5 days before any symptoms were visible in controls.

Human Studies: In patients receiving artificial fever, studies were made on Vitamin C excretion in urine and on its concentration in the blood. Urinary Vitamin C excretion was determined the day before fever treatment, the day of treatment and the day following treatment. Blood Vitamin C was determined before treatment and immediately after treatment. A modification of Bessey and King's[4] method was used to determine Vitamin C in urine; and the method of Pijoan and Klemperer[6] was used to determine it in blood plasma.

Because of difficulty in collection of complete 24-hour samples of urine from patients studied, reliable results were obtained on only 4. Two of these had previous fever treatment, and each excreted 17 mg of Vitamin C the day before treatment. Each of the others excreted 23 mg. The day including the fever treatment, none excreted more than 10 mg. Following fever, the first 2 excreted 8.0 and 15.0 mg, the last 2 each excreted 16 mg. Since Vitamin C is excreted in sweat[7, 8] no significance can be attached to the low values obtained during fever. In all cases, however, excretion was lower following fever than before.

Since excretion did not return immediately to pre-treatment level and those individuals who had previous treatments were excreting very little Vitamin C (normal excretion in 24 hours is 25 mg or more[9]), lowering of Vitamin C stores is indicated. Whether fever increases the physiological need for Vitamin C or induces an excessive loss in sweat cannot be determined by this type of experi-

6 Pijoan, M., and Klemperer, F., *J. Clin. Invest.*, 1937, **16**, 443.

7 Cornbleet, Theodore, Klein, R. I., and Pace, E. R., *Arch. Dermatol., Syphilol.*, 1936, **34**, 253

8 Lilienfeld, A., Wright, I. S., and MacLenathen, E., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 184.

9 Youmans, John B., Corlette, M. B., Akeroyd, J. H., and Frank, H., *Am. J. Med. Sciences*, 1936, **191**, 319.

ment. However, when the results with guinea pigs are considered, an increased physiological need for Vitamin C during fever is indicated.

Studies on blood were interesting but did not aid in judging Vitamin C utilization. It seemed that the blood Vitamin C concentration should be reduced during fever if an excessive amount was lost in sweat, or tissues required more than normal. However, a decrease was not observed in any of 6 patients studied. In all of them, values after treatment were equal to or greater than the values before treatment which indicates that Vitamin C, like blood chlorides and blood sugar,[10] concentrates with the concentration of blood during fever.

Summary. Studies of Vitamin C excretion before and after periods of artificial fever show that fever increases the Vitamin C requirement of man. Studies of Vitamin C stores in adrenals and kidneys of guinea pigs show that artificial fever increases the requirement or accelerates the destruction of Vitamin C. Since guinea pigs cannot lose Vitamin C in sweat, an increase in the physiological need for Vitamin C during fever is indicated.

10159

Localization of the Neural Inductor and Tail Mesoderm in a Frog Egg (*Hyla regilla*)*

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The Neural Inductor. In the urodeles the dorsal and lateral blastoporal lips of the early gastrula induce a secondary neural plate when implanted under presumptive epidermis.[1, 2] Although in *both* urodeles and anurans the anlagen of chorda and somites occupy the dorsal and lateral lips,[3] it has been reported that in anurans the neural plate inductor is limited to the dorsal lip.[4] Is this a general characteristic which distinguishes the anuran from

10 Krusen, Frank H., *Am. J. Med. Sciences*, 1937, **193**, 470.

* Aided by a research grant from the University of California.

1 Bautzmann, H., *Arch. f. Entw.-mech.*, 1926, **108**, 283.

2 Schechtman, A. M., *Univ. Calif. Publ. Zool.*, 1934, **39**, 277.

3 Vogt, W., *Arch. f. Entw.-mech.*, 1929, **120**, 384.

4 Schmidt, G. A., *Zool. Anzeiger*, 1936, **116**, 323.

the urodele egg? Towards the solution of this problem a study was made of the neural inductor in the anuran *Hyla regilla.*

The jelly and egg membranes were removed from early gastrulæ with steel needles. After washing the eggs in 3-4 changes of Holtfreter solution, the distorted eggs were discarded. The remaining more or less spheroidal eggs were then operated upon, using glass needles drawn out at the end into fine filaments so as to permit cutting with a minimum of cell destruction. Various portions of the mesodermal girdle (and some of the adjacent ectoderm since it is quite impossible to ascertain the exact boundary between ectoderm and mesoderm) were removed and immediately placed into the blastocœles of early gastrulæ. In the discussion below the portions of mesoderm used are designated by degrees. The dorsal mid-line (running through the center of the dorsal lip) is 0°, the center of the ventral lip is 180°, and the center of each lateral lip is 90. Dorsal lip implants, which include the mesoderm as far as 45° on either side of the dorsal mid-line, induced in almost 100% of the cases. Mesoderm in the region between 45° and 80° induced in only 17-20% of the cases. Mesoderm located 55° or more from the dorsal mid-line showed no inductive capacity whatsoever.

These results show that the neural inductor is limited to the dorsal mesoderm extending about 45° on either side of the mid-line, as Schmidt reported,[4] which is about half the meridional extent of the neural inductor in the urodeles, *Triton* and *Triturus.*

The Tail Mesoderm. In the course of the above work it was noted that in some eggs cultured for 72 hours (most were kept only 24-30 hours) the induced structures never included a tail or tail-like structure. This was unexpected since in both the anurans and the urodeles the tail-forming material is considered to extend completely around the equatorial region of the egg.[3] In agreement with this concept the dorsal blastoporal lip of the *urodeles* induces not only a neural tube but in many instances a secondary tail as well.[5, 6, 7] However, in the anurans *Rana* and *Pelobates* Schmidt[4] found tail-like projections were produced only by lateral parts of the mesoderm.

In the present experiments on *Hyla* the operative method was the same as that described above, but the eggs were cultured at least 72 hours, many for twice this period, in order to allow ample time for tail development.

The dorsal lip mesoderm, extending as far as 45° from the dor-

5 Lehmann, F. E., *Arch. f. Entw.-mech.*, 1932, **125**, 566.

6 Holtfreter, J., *Arch. f. Entw.-mech.*, 1936, **134**, 466.

7 Reith, F., *Z. f. wiss. Zool.*, 1937, **150**, 179.

sal mid-line, did not produce a tail in a total of 23 cases tested. Horn-like extensions were numerous and resembled those already described for urodeles (*e. g.,* Lehmann[5] and Reith[7]). As pointed out by Lehmann[5] they are not to be confused with true tails. They are simply chorda-like elongations of the implanted dorsal lip and are readily obtained by culturing dorsal lips *in vitro*.

True tails, corresponding to the tails in Harrison's stages 33-36, were obtained after implanting portions of the lateral and ventro-lateral mesodermal regions (the mesoderm between 45° and 155°). The portions of this mesodermal region closer to the mid-dorsal line were clearly less effective than the more distant region in forming secondary tails. Thus the lateral lip mesoderm (located between 45° and 115°) developed into tails in 8 out of a total of 17 cases, while the ventro-lateral mesoderm (90°-155°) yielded tails in 5 out of 6 cases. These tails resembled the normal tail in their tapering shape, the slight lateral flattening, and the presence of one or two fin rudiments in some but not all of the specimens.

Mesoderm from the region of the ventral lip (160°-200°) yielded no tails in a total of 13 specimens. The implants at first formed low hillocks which suggested possible early tail buds but these hillocks remained low or regressed to irregular masses. When some ventro-lateral mesoderm was included with the ventral mesoderm (135°-225°) tails were formed in 18 out of a total of 25 cases.

The above results with implants were checked with extirpation experiments, in which specific regions of the mesoderm were removed from early gastrulæ. Tails were formed in the absence of any one of the following regions: dorsal lip, lateral lips, ventro-lateral lips, ventral lip, dorsal plus lateral lips, ventral plus ventro-lateral lips. Tailless embryos resulted only when both the lateral and the ventro-lateral lips were removed from the same egg. In a total of 8 eggs all the mesoderm except the dorsal lip was removed. These eggs developed only a head in most cases although 2 specimens also formed the upper part of the trunk. The heads were quite normal in size with two suckers and a stomodeal pit.

These results show that the potency for tail formation is more restricted in the anuran *Hyla* than in the urodeles. We could get no evidence that the dorsal and the ventral lip mesoderms had any tail potency at all. Furthermore the potency for tail formation is greater in the ventro-lateral mesoderm than in the lateral mesoderm. We may interpret the results as indicating the existence of two separate anlagen for the tail, separated from one another by the

dorsal and the ventral lips. It is, however, also possible that the tail-mesoderm forms a continuous girdle around the equator of the egg as in the urodeles but that the portions in the dorsal and ventral lips are so attenuated as to be unable to assert their normal developmental traits. In either case the conclusion must remain that tail-potencies are largely concentrated in two bilaterally located regions of the mesoderm.

Conclusions. 1. In the early gastrula of the anuran *Hyla regilla* the inductor of the neural plate is localized approximately in the dorsal lip region of the mesodermal band, that is, it extends about 45° on either side of the dorsal mid-line. 2. The tail-forming potencies are localized in the lateral and ventro-lateral regions of the mesoderm, with the higher percentage of tails developing from the ventro-lateral regions after implantation. The dorsal and the ventral lip mesoderm do not form tails after implantation. 3. The present results show that the anuran *Hyla* has distinct differences in the localization of certain developmental capacities as compared to the eggs of urodeles. Both the neural inductor and the tail mesoderm have a more limited meridional extent in *Hyla.*

10160

Effect of Fat on Rate of Availability of Orally Ingested Carbohydrate.

HERBERT POLLACK AND HENRY DOLGER.

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In a recent publication[1] it was shown that the ingestion of rapidly available carbohydrates by diabetic patients using protamine zinc insulin, resulted in rapid rises and precipitous drops in blood sugar concentration. Such marked fluctuations in blood sugar concentration are no doubt responsible for many of the difficulties encountered in the clinical management of diabetes with protamine zinc insulin. A solution of this problem presents itself in the well-known property of fats in delaying gastro-intestinal activity. To test this hypothesis, 25 patients were given a standard amount of carbohydrate with and without fat. The banana was chosen because

1 Pollack, H., and Dolger, H., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 577.

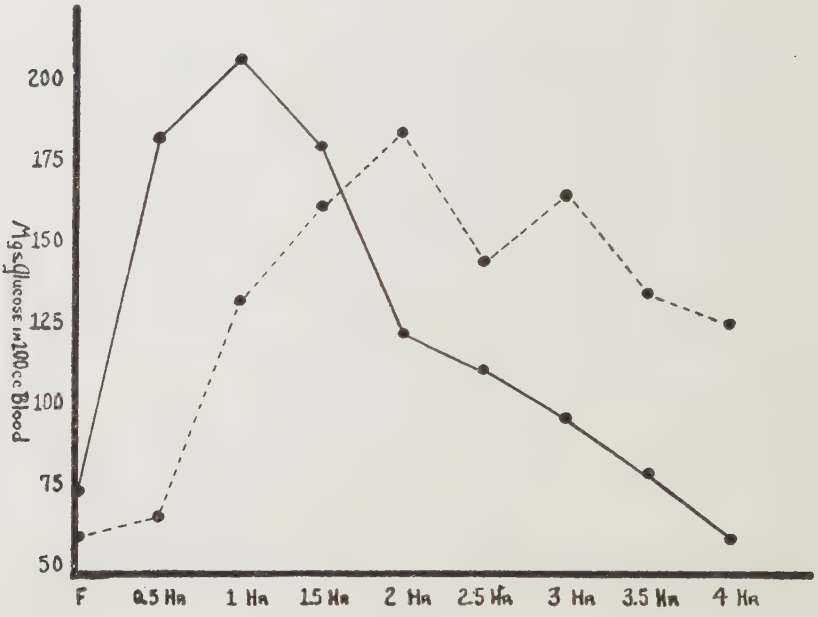

FIG. 1.
Curve after banana and cream .----.----.----.
Curve after straight banana .——.——.——.

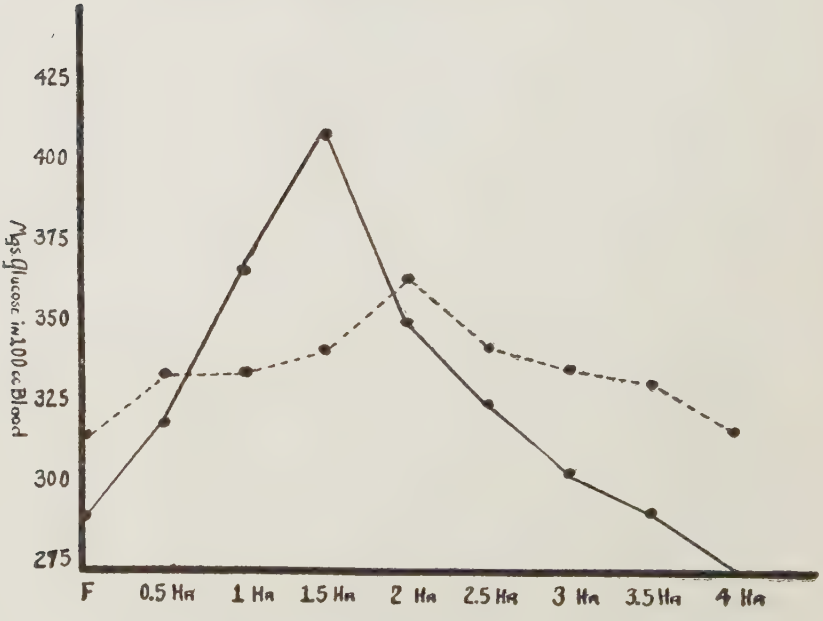

FIG. 2.
Curve after banana and cream .----.----.----.
Curve after straight banana .——.——.——.

of its universal availability, standard composition, palatibility, and ability to be combined with cream.

The patients chosen for this experiment were severe diabetics maintained with protamine zinc insulin exclusively. The tests were carried out in the post-absorptive state, and 24 hours after the last injection of protamine zinc insulin. Blood sugars were determned by the Somogyi modification of the Schaffer-Hartmann method at ½ hourly intervals for 4 hours. Each patient received 220 g of banana alone for the preliminary studies, and after 10 days the same amount of banana with 4 ounces of 30% cream.

Within certain variations all patients responded in the same way. Three representative curves were chosen for illustration to show the results and the variations. There are 2 major advantages of the fat-carbohydrate mixture over the carbohydrate alone. The first is the delay in the time of maximum absorption for from 1 to 2 hours. The second is the elimination of the precipitous drop in blood sugar concentration seen with carbohydrate alone.

The advantages of these fat-carbohydrate mixtures when protamine zinc insulin is being used are quite obvious. The activity of the protamine zinc insulin is not manifested for several hours after its injection, hence the importance of delaying the post-

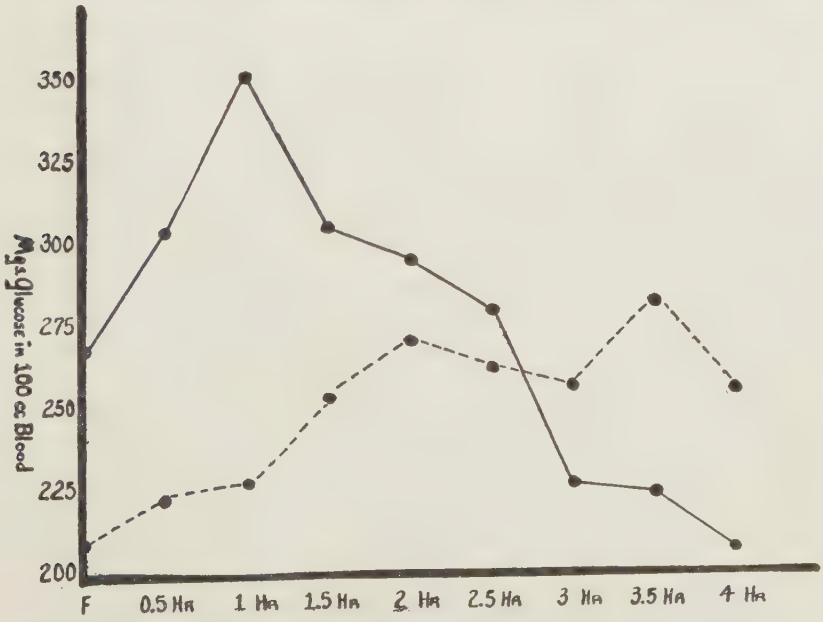

FIG. 3.
Curve after banana and cream .----.----.----.
Curve after straight banana .——.——.——.

prandial rise in blood sugar. This delay may allow the absorptive period to come within the range of insulin activity of the current injection.

The sustained absorption seen with the fat mixtures tends to prevent the disturbing post-hyperglycemic hypoglycemias previously described.

10161

Protein as a Source of Carbohydrate for Patients Using Protamine Zinc Insulin.

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The introduction of protamine zinc insulin for the management of diabetes gave rise to several difficulties. Some of these, with suggested solutions, have been discussed in previous papers.[1, 2, 3] In addition, the prolonged action of protamine zinc insulin during the whole 24-hour period led to severe nocturnal hypoglycemic episodes. The recommendation by Conn and Newburgh[4] of high protein diets for the treatment of spontaneous hypoglycemia can be applied in principle to this problem. It is well known that about 50% of protein is available to the body as carbohydrate. It is also known that the gastric-emptying time with a meat meal may be prolonged to 3 or more hours. The time of small intestinal digestion, absorption, transportation to the liver, deaminization, and synthesis of glucose further delays this availability.

To test this hypothesis, 40 diabetic patients were given 400 g of lean chopped beef, lightly broiled. This test meal was given 24 hours after the last dose of insulin (protamine zinc insulin) on a fasting stomach. Blood sugars were taken at one-half hourly intervals for a period of 4 hours, as previously described. Control experiments consisted in blood sugar determinations on the same patients under similar conditions, but without any food.

The results were uniform in all patients. There is a slow but definite rise in blood sugar concentration throughout the whole experi-

1 Pollack, H., and Lande, H., *N. Y. State J. Med.*, 1938, **38**, 1.

2 Pollack, H., *J. Mt. Sinai Hosp.*, **4**, 437.

3 Pollack, H., and Dolger, H., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 577.

4 Conn, J. W., and Newburgh, L. H., *J. Clin. Invest.*, 1936, **15**, 665.

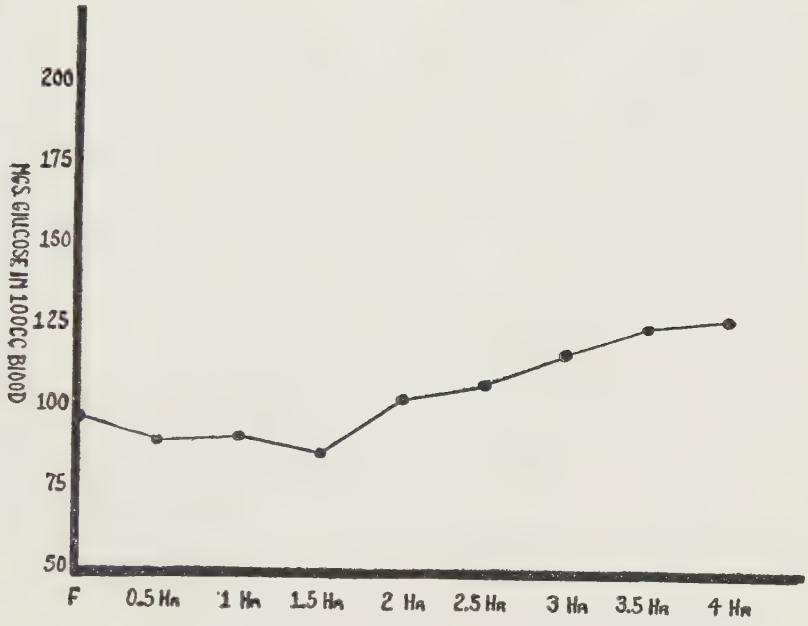
200
175
150
125
100
75
50
MGS GLUCOSE IN 100CC BLOOD
F
0.5 Hr
1 Hr
1.5 Hr
2 Hr
2.5 Hr
3 Hr
3.5 Hr
4 Hr

FIG. 1.

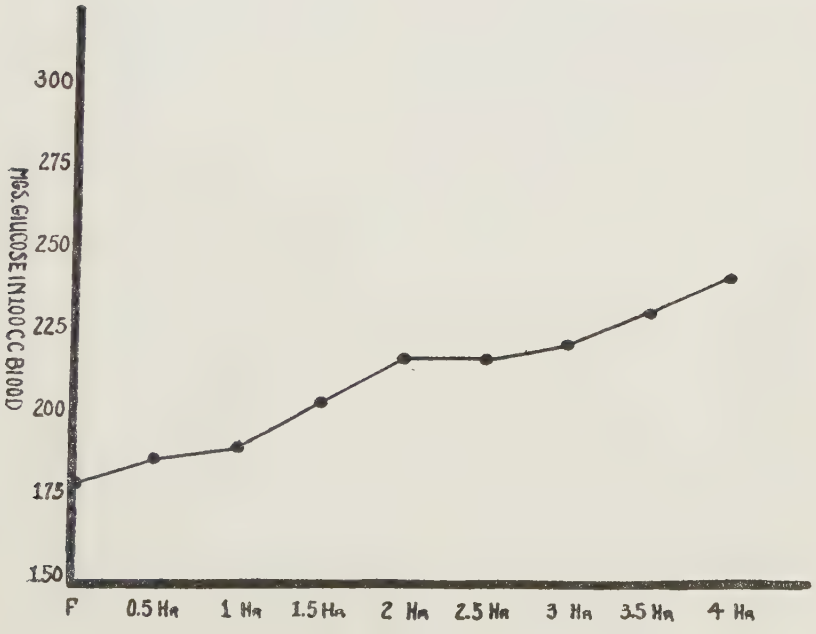
300
275
250
225
200
175
150
MGS GLUCOSE IN 100CC BLOOD
F
0.5 Hr
1 Hr
1.5 Hr
2 Hr
2.5 Hr
3 Hr
3.5 Hr
4 Hr

FIG. 2.

mental period. In contrast to the curves obtained after orange juice, banana, or banana and cream, previously published, and bread, oatmeal and potato, there is absolutely no compensatory drop in blood sugar level. Curves 1 and 2 are representative examples of this series of experiments with their controls.

The application of this principle to the problem of protamine zinc insulin therapy has eliminated nocturnal hypoglycemia. Patients are instructed to take 50% or more of the daily protein allowance at the evening meal, in order to buffer the tendency toward decreasing blood sugar concentration during the night.

10162 P

Syndromes Secondary to Prolonged Hypoglycemia.*

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In an attempt to study the effects of prolonged hypoglycemia dogs and cats were injected with insulin and subjected to coma for varying periods. The duration of the coma was frequently extended as long as possible compatible with life. The symptomatology, which subsequently developed, occurred despite a blood sugar raised to normal levels or higher by the administration of sugar. The post hypoglycemic syndromes are characterized by their variability. For example, dog No. 4, which received insulin for 4 days and was intermittently in coma for approximately one-half of that time, became totally blind in his left eye and retained only light perception in his right one. He was continually moving about and in his aimless restlessness would bump into any object that might confront him. When food was placed in his mouth he showed no inclination to swallow it, but kept it there indefinitely. Another animal, cat No. 2, remained in a fixed position, an awkward one, standing on his hind legs and leaning upright against the side of the cage for at least 12 hours, though the blood sugar was raised to normal levels throughout this period.

Cat No. 6 displayed the crossed extensor reflex and spasticity of the posterior extremities. In cats the temperature regulation is frequently impaired after prolonged hypoglycemia. Cat No. 3 became

* Aided by a grant from the Child Neurology Research (Friedsam Foundation).

to a great extent poikilothermic. After recovery from a 7-hour period of hypoglycemia his temperature was only 36.6° despite torrid weather. The next morning his temperature was 38.7°, but after remaining in the ice room for 15 minutes his temperature fell to 38° and 20 minutes later to 36.3° with no shivering. Four hours after removal from the ice room the animal's temperature rose to 39° and his response to temperature was not tested again. Throughout this period and for several days thereafter the animal remained somnolent, did not respond to stimuli, nor did he swallow the food which was placed in his mouth. Such a preparation is termed "vegetable". Bradycardia is a frequent occurrence during hypoglycemia and this bradycardia in many instances continues for several hours after the administration of sugar. If the survival can be prolonged for several days, partial or complete recovery may be observed. Sudden death may also occur.

Marked hypoglycemia depresses the metabolism of the brain.[1, 2, 3] These preliminary experiments reveal that if this depression of cerebral metabolism is too prolonged in many instances irreversible cerebral changes occur. These changes are not restricted to any given area but may affect the various parts of the brain. This has also been disclosed in histological studies. Somnolence and temperature regulation have been localized as functions of the hypothalamus. Bradycardia, too, may be due to a release of parasympathetic centers in that region. The restlessness which developed in dog No. 4 resembled that of a decorticate animal, while the spasticity and crossed extensor reflex is like that of a decerebrate cat (No. 6). The catatonia, too, may be related to the disturbance in the functions in the brain. Blindness as well as these other changes may also result from nitrous oxide anesthesia, thus emphasizing the similarity between the effects of anoxia and those of glucose deprivation.[4, 5, 6] In view of results such as desribed above it is important to note that patients receiving the insulin treatment for schizophrenia should not be subjected to too prolonged hypoglycemia.[7]

1 Dameshek, W., and Myerson, A., *Arch. Neurol. and Psychiat.*, 1935, **33**, 1.

2 Himwich, H. E., and Fazekas, J. F., *Endocrinology*, 1937, **21**, 800.

3 Himwich, H. E., Bowman, K. M., Wortis, J., and Fazekas, J. F., *Science*, 1937, **86**, 271.

4 Weil, A., Liebert, E., and Heilbrun, G., *Arch. Neurol. and Psychiat.*, 1938, **39**, 467.

5 Himwich, H. E., Bowman, K. M., Fazekas, J. F., and Orenstein, L. L. PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 359.

6 Gellhorn, E., *Arch. Neurol. and Psychiat.*, 1938, **40**, 125.

7 Sakel, M., *The Pharmacological Shock Treatment of Schizophrenia*, Nervous and Mental Disease Publishing Co., 1938.

10163 P

Reciprocal Oxygen Changes on Both Sides of Placenta During Uterine Contraction and Relaxation.

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Attention has been directed to the intermittent character of intrauterine fetal respiratory movements and it has been suggested that prelabor uterine contractions may be related to this phenomenon.[1] Other motor activities of unanesthetized fetuses delivered with the placental circulation intact varied with contraction and relaxation of the uterus. With each contraction, reddening of umbilical veins and corresponding improvement of fetal color was observed. Uterine relaxation led to darkening of the blood. It was evident that alteration of respiratory gas content of the fetal blood was related to the fluctuations in fetal activities. The umbilical vein blood was richer in oxygen during uterine contraction than during relaxation.[2] Do these changes occur normally *in utero* or are they, perhaps, the result of operative interference with the placental exchange mechanism? The present experiments were planned to help determine the answer.

Large superficial tributaries of the uterine veins carry blood from the maternal side of the placentæ of the cat. It was possible to obtain 0.2 cc samples of blood principally from the placenta from these vessels for analysis by the Van Slyke and Neill[3] micro-manometric method for combined carbon dioxide and oxygen determinations. The cats were not anesthetized but had been decerebrated by tying the carotid and basilar arteries an hour or more before. The uteri were exposed after immersing the animals in a constant temperature bath of Locke's solution.

If the undisturbed fetuses are removing more oxygen from the maternal blood during uterine contraction than during relaxation, the blood leaving the placenta on the maternal side should be lower in oxygen in the period of contraction. Estimations of the blood oxygen are presented in the Table I. The placental samples were

* Aided by a research grant from the American Academy of Arts and Sciences. Technical assistance of Mrs. C. Holm, Miss D. Nelson, Mr. C. L. Bishop, and Mr. Q. B. DeMarsh is gratefully acknowledged.

1 Windle, W. F., Monnier, M., and Steele, A. G., *Physiol. Zool.*, 1938, **11**, 425.

2 Windle, W. F., and Steele, A. G., 1938, *J. Physiol.*, in press.

3 Van Slyke, D. D., and Neill, *J. Biol. Chem.*, 1924, **61**, 523.

taken as close to one another as possible but the maternal femoral arterial and the umbilical blood samples were drawn earlier and later; often 30 minutes or more elapsed between them. The values for carbon dioxide fluctuated widely, depending upon variations in maternal respiratory conditions,[4] and can not serve so well as the oxygen estimations for comparison. In most of the experiments, it was found that the maternal placental blood contained less oxygen

TABLE I.
Oxygen Content in Vol. % of Blood.

Cat		Fetus		Placenta	Maternal artery	Maternal vein from placenta				Umbilical vein	
No.	Age days	No.	wt g	wt g	O_2	C → R O_2	R O_2	R → C O_2	C O_2	R O_2	C O_2
1	23	2	0.2	2.0	19.4		10.0		8.6		
									9.9		
3	35	1	4.0	10.0	14.2		6.8*		5.1		
							10.3				
4	38	1	6.5	11.0	13.2		3.2	2.4	2.7		
6	—	1	16.0	14.0	13.5	2.9	6.3		4.6		
		2	18.0	14.0			9.2†			1.0	
		3	17.0	17.0							4.0
7	—	3	19.0	20.0	13.3	1.7	6.2	6.6			
							7.0				
8	46	1	15.0	10.0	18.5	7.2	5.9*		7.5		
		2	19.0	13.0					4.7		
10	46	1	17.0	24.5‡	14.5		7.1		5.5		
		3	23.5	15.5			6.2		3.3		
11	48	1	25.5	15.5	15.9	7.0	9.0		8.7	4.9	
		2	27.5	17.0		9.0	9.0				
		3	31.0	15.0							8.1
12	—	2	29.0	17.0	13.3				2.8		
									7.9†		
15	—	1	30.9	14.0	13.4		5.6				
		4	45.7	17.4		4.2	6.5			3.7	8.4
23	—	1	77.0	23.0	11.0		2.9	3.5		4.1	1.6
24	—	1	78.0	17.0	11.2	2.8					
		3	75.0	18.0		4.5		3.9			
26	—	1	103.0	25.0	10.5		6.6		1.2		
		2	90.0	15.0		0.2		1.9			
27	—	1	104.0	22.0	12.2		2.7		1.1	1.8	2.5
		2	100.0	20.0			1.2		0.6‖		
28	—	1	99.0	20.0	—	5.0	5.9				7.7
							8.7				
		2	106.0	27.0	12.0§					5.3	8.1

* = Hypertonic uterus; † = umbilical cord occluded; ‡ = placenta serving twin fetuses; § = oxygen capacity; ‖ = under pitocin.

when the uterus was contracted (C) or when a contraction wave was passing (C → R) than it had during relaxation (R) or at the very beginning of a contraction (R → C). It was sometimes very

[4] Steele, A. G., and Windle, W. F., 1938, *J. Physiol.*, in press.

difficult to determine the state of uterine activity exactly, especially when the contractions were not very strong.

During a few experiments not presented in the table, the uterus lacked tonus and conditions suggesting shock obtained; all venous blood was reduced. Hypertonicity of the uterine muscle apparently favored the placental exchange of oxygen (see cats 3 and 8) but when the uterus was tightly contracted, as after pitocin injection, blood vessels were occluded.

That fluctuation in the gas content of placental blood may be related to the amount of gas taken up by the fetus was indicated by experiments in cats 6 and 12. The gas values marked by the dagger (†) were obtained from blood samples drawn from distended uteri but after clamping the umbilical cords of the fetuses. In cat 6, the blood passing back to the mother from the placenta and fetus contained 6.3 volumes % oxygen but that returning from the placenta only contained 9.2 volumes %. Similarly in the other experiment results were 2.8 and 7.9 volumes %. It will be seen that these fetuses obtained about 2.9 volume % oxygen during relaxation and 5.1 volume % during contraction of the uterus.

10164 P

Adsorption of Heterophile Antibody by Pneumococci of Different Types.

H. M. POWELL AND W. A. JAMIESON.

From the Lilly Research Laboratories, Indianapolis, Indiana.

It has been shown[1] that a large proportion of strains of pneumococci of types I, II, and III contain heterophile antigen conforming to the Forssman characteristics. Experimental data establishing this point comprise both the results of active immunization of rabbits, and of adsorption of the antibody under controlled conditions *in vitro*. It has become of interest to test by the adsorptive method the heterophile-antigen content of pneumococcal strains of the higher type-numbers since the rapid typing methods have identified these types on a scale sufficiently large to merit close study of both species-antigens and type-antigens in more detail.

Heterophile antibody was prepared in rabbits by 6 intravenous injections of the usual increasing doses of heat-killed pneumococci from culture DRI, and R-variant of the Neufeld type I strain. The

[1] Bailey, G. H., and Shorb, M. S., *Am. J. Hygiene*, 1931, **13**, 831.

serum obtained a week after the last dose was heated at 56°C for 30 minutes and tested for its content of antisheep hemolysin according to the technic heretofore described.[2] The unit of sheep erythrocytes was 0.1 cc of a suspension made by adding one part of washed red cells of whole-blood concentration to 3 parts of salt solution; the dose of complement was 0.1 cc of fresh 20% guinea-pig serum.

This heterophile serum was then diluted so that 2 cc contained 50 units of lysin; 2 cc amounts were then adsorbed at 37°C for 30 minutes with 25 billion sedimented boiled pneumococci of different strains and types. The suspensions were then sedimented and the supernatant fluids, along with unadsorbed controls, were tested for their content of antisheep lysin. This test was conducted in 2 stages since the adsorbed supernatant fluids were often anticomplementary. The first stage comprised addition of a unit of sheep red cells to different concentrations of the supernatant fluids, incubation at 37°C for 30 minutes, and resuspension of the sedimented cells in fresh salt solution. This was followed by the addition of complement and reincubation for one hour. The number of units of antibody adsorbed out of a total of 50 was then computed as the original unitage minus the final unitage.

TABLE I.
Adsorption of Heterophile Antibody by Pneumococci of Higher Type Numbers.

Pneumococcus type	No. units heterophile antibody adsorbed out of 50 unit total	Pneumococcus type	No. units heterophile antibody adsorbed out of 50 unit total
4	0	14L	+45
5	40	15	45
6A	40	17	45
6B	0	18	40
7	40	20	+45
8	45	21	40
9	40	22	+45
10	45	24	+45
11	0	27	+45
12	45	31	0
13	+45	32	45
14F	45	"DRI"	45

Table I shows the results of these tests. All types* except 4, 6B, 11, and 31 adsorbed a high percentage of the 50 units of heterophile antibody to which they were exposed. Of the 4 non-adsorbing strains, 6B is said to represent the older Cooper type 26. In our hands, however, culture 6B but not 6A has agglutinated with type 6

2 Powell, H. M., *J. Immunol.*, 1926, **12**, 1.

* These were obtained through the courtesy of the National Institute of Health.

commercial typing serum. Our results showing that 19 of 23 separate group 4 types readily adsorb heterophile antibody may be compared with those of Bailey and Shorb,[1] showing that 32 of 35 "type IV" strains adsorb heterophile antibody. In all probability, however, Bailey and Shorb used larger adsorbing doses of pneumococci than we did.

In addition to the tests herewith reported we have had occasion to test the heterophile-antibody-adsorbing capacity of several pneumococcal strains belonging to types 1, 2, and 3. The type 1 strains have adsorbed a large amount, the type 2 strains a very small amount or none, while the type 3 strains adsorbed an intermediate amount of heterophile antibody. These results agree in the main with those reported by Bailey and Shorb.[1]

Conclusion. Heterophile antigen has a wide distribution in the various types of pneumococci. It is logical to assume, therefore, that heterophile antigen is at least part of the complex species-antigenic structure of most pneumococci.

10165

Excretion of Mercury Following Administration of Mercurial Diuretics with and without Theophylline.

ARTHUR C. DEGRAFF, ROBERT C. BATTERMAN, ROBERT A. LEHMAN AND ELTON YASUNA.

From the Department of Therapeutics, New York University College of Medicine.

It has recently been shown that the addition of theophylline to the mercurial diuretics Mercurin and Salyrgan practically prevents their local toxic action[1, 2] and promotes their absorption after intramuscular injection.[3] Although Mercupurin (Mercurin with theophylline) has been found to have a somewhat greater diuretic efficiency than Salyrgan,[4-7] it remains to be proven unequivocally, however,

[1] DeGraff, A. C., and Batterman, R. C., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1546.

[2] DeGraff, A. C., Batterman, R. C., and Lehman, R. A., *ibid.*, 1938, **38**, 373.

[3] DeGraff, A. C. Batterman, R. C., and Lehman, R. A., *J. Pharm. Exp. Therap.*, 1938, **62**, 26.

[4] Thompson, W. A. R., *Quart. J. Med.*, 1937, **30**, 321.

[5] Crawford, J. H., and McDaniel, W. S., *Ann. Int. Med.*, 1935, **8**, 1266.

[6] Fulton, M. N., and Bryan, A. H., *J. Lab. Clin. Med.*, 1935, **20**, 1252.

[7] DeGraff, A. C., Nadler, J. E., and Batterman, R. C., *Am. J. Med. Sci.*, 1936, **191**, 526.

that theophylline exerts any influence after the drug has reached the circulation. It was therefore decided to study the excretion of mercury by laboratory animals after the administration of these diuretics with and without theophylline. It is fully realized that results thus obtained cannot necessarily be referred to man. Nevertheless, it was felt that information concerning the pharmacology of drugs of this type could more readily be obtained by using animals whose food, salt and water ingestion could be kept perfectly constant.

A total of 24 albino rabbits weighing between 2 and 3 kg were used in these experiments. All the animals had been raised on a diet of alfalfa hay and oats and had received a constant daily supply of water. On the day of the experiment each rabbit was tied to an animal board and 75 cc of water at body temperature administered by stomach tube. One hour later the animal was catheterized (French No. 10 catheter), the urine discarded and 0.10 cc of the drug to be studied was injected with a tuberculin syringe and 26 gauge needle either into the tibialis anterior muscle or one of the marginal veins of the ear. This was equivalent to 3.71 mg of mercury in the case of Salyrgan or Salyrgan with theophylline and 3.80 mg in the case of Mercurin or Mercupurin. The catheter was strapped in place with adhesive tape and all the urine excreted within the next 6 hours collected in 25 cc graduates. Since the rates of excretion vary considerably for the different drugs and methods of administration, it was necessary to space the collection of samples so that the time at which maximum excretion occurred could easily be determined. The mercury content of each sample of urine was then determined by the method of Winkler,[8, 9] modified by Gettler and Lehman.[10]

The protocols are given in condensed form in Table I and the results are summarized in Fig. 1 and Table II. In Fig. 1 is shown the influence of theophylline upon the total amount of mercury excreted in the urine of the rabbit within 6 hours. The experiments were not continued beyond this point since in all cases the amount of mercury in the urine dropped during the last hour of the period to less than 5% of that administered. Three bars are plotted for each drug and represent the values obtained from 3 animals treated identically. It will be seen that the variation between animals is

8 Winkler, W. O., *J. Assn. Off. Agric. Chemists*, 1935, **18**, 638.

9 Winkler, W. O., *ibid.*, 1936, **19**, 233.

10 Gettler, A. O., and Lehman, R. A., *Am. J. Clin. Path.*, Tech. suppl., 1938, **8**, 161.

moderate and that the trend is unmistakable. Theophylline increases the total mercury excretion 30 to 40% after intravenous injection and 100 to 300% after intramuscular injection. The effect is so much greater by the intramuscular route because of the slower absorption of the preparations not containing theophylline. In fact it has been shown[3] that while, on the average 55% of the injected Salyrgan, and 57% of the Mercurin, are absorbed within the first 6 hours, Mercupurin and Salyrgan with theophylline are completely absorbed during the same time interval. It would be more logical,

TABLE I.
Mercury Excreted After Various Time Intervals.

Time after injection	Mg. of mercury found in urine*			
	Mercurin		Salyrgan	
	intravenous	intramuscular	intravenous	intramuscular
min.				
15	.374	—	.468	—
30	.389	.055	.321	.028
45	.619	—	.587	—
60	.481	.112	.361	.084
90	.399	.101	.290	.095
120	.244	.131	.106	.075
150	—	.088	—	.064
hr.				
3	.157	.070	.150	.047
4	.069	.150	.083	.078
5	.020	.112	.032	.050
6	.048	.152	.063	.046
	Mercupurin		Salyrgan with theophylline	
	intravenous	intramuscular	intravenous	intramuscular
min.				
7	.308	—	.475	—
15	.671	.181	.898	.054
22	.518	—	.695	—
30	.327	.539	.442	.867
45	.483	.455	.539	.414
60	.384	.579	.216	.554
90	.365	.319	.228	.412
120	.292	.306	.111	.223
150	—	.158	—	.105
hr.				
3	.164	.189	.120	.256
4	.118	.140	.097	.123
5	—	.091	.052	.112
6	.041	.059	.060	.132

*Each value represents the average of 3 experiments.

therefore, to express the excretion of Mercurin and Salyrgan as percentage of absorbed mercury which is excreted, rather than percentage of administered mercury. This calculation was made and its effect is shown graphically by the dotted lines in Fig. 1. It will be seen that theophylline still distinctly increases the total urinary ex-

TABLE II.
Maximum Excretion Rates.

Drug	Intravenous				Intramuscular			
	Mercurin	Mercupurin	Salyrgan	Salyrgan with theophylline	Mercurin	Mercupurin	Salyrgan	Salyrgan with theophylline
Max. excretion rates in % of administered mercury excreted per min.*	1.2	2.4	1.2	3.0	0.13	0.72	0.09	1.2
Min. after injection at which max. excretion rates occur.*	33	14	43	14	85	43	75	25

*Mean of 3 experiments.

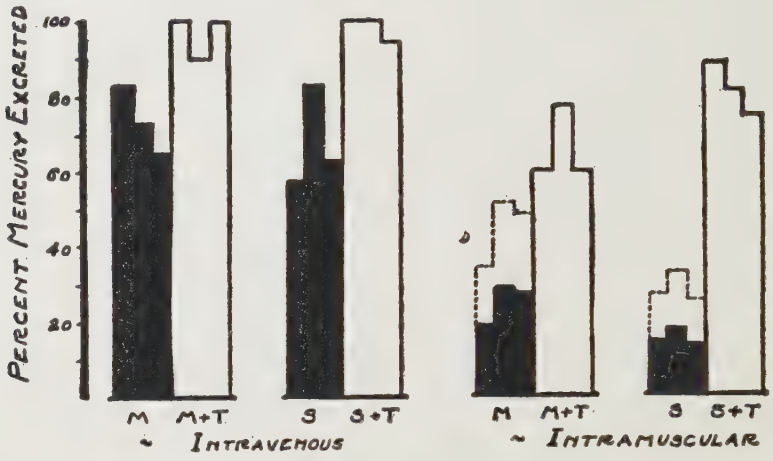

FIG. 1.

Diagrams showing the influence of theophyline upon the percentage of the injected mercury which is excreted in the urine of the rabbit within 6 hours. Dotted lines show the percentage of absorbed mercury which is excreted in the cases where absorption is incomplete. Abbreviations: M—Mercurin; M + T—Mercurin with theophylline or Mercupurin; S—Salyrgan; S + T—Salyrgan with theophylline.

cretion of mercury. By taking this factor into account it is possible to conclude that theophylline continues to influence the action of mercurial diuretics after their absorption into the blood.

It necessarily follows that if the total mercury excreted is increased by theophylline, then the rate of excretion must also be increased. The excretion rates over the time intervals given in Table I were calculated and the averages of the 3 maximum values for each 3 identical experiments are listed in Table II.* The average time after injection at which these maxima occur is also given and it will be seen that theophylline increases the mean maximum excretion rate to the same relative degree as it increased the total mercury excretion, while at the same time it causes the maxima to occur somewhat sooner.

Summary of Results. Theophylline was found to influence the urinary excretion of mercury following the injection of Mercurin or Salyrgan in the following ways: 1. The percentage of the administered mercury which is excreted within 6 hours increased 30 to 40% after intravenous injection and 100 to 300% after intramuscular injection. 2. The maximum rates of excretion increased greatly in all cases and by all methods of administration. 3. The maximum excretion rates occurred somewhat earlier.

* This procedure is used instead of determining the peak of the excretion rate curve since it requires no subjective estimate.

The results demonstrate that the combination of theophylline with these mercurial diuretics modifies their action after absorption as well as before.

10166 P

Recovery in the Rat from the Diabetes Insipidus Caused by Posthypophysectomy.

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Richter[1] and Pencharz, Hopper and Rynearson[2] have shown that after removal of the posterior lobe of the hypophysis ("posthypophysectomy") of the rat. with minimal damage to the anterior lobe, there ensues a profuse diabetes insipidus. Both were of the opinion that the condition was permanent; Richter followed the water intake of his rats for about 60 days and Pencharz, *et al.*, for 100 days. White,[3] using rats posthypophysectomized by Pencharz, reported 3 animals to be clearly diuretic a year after operation. In our experience, however, recovery from the diabetes insipidus produced by the same operation customarily occurs in time. Fig. 1 shows the

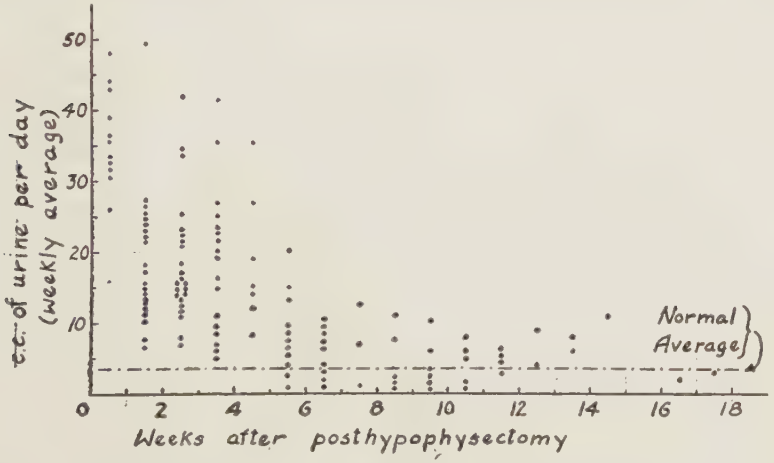

FIG. 1.
Urinary excretory rates after posthypophysectomy in the rat.

1 Richter, C. P., *Am. J. Physiol.*, 1934, **110**, 439.

2 Pencharz, R. I., Hopper, J., and Rynearson, E. H., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 14.

3 White, H. L., *Am. J. Physiol.*, 1937, **119**, 5.

excretory records of a group of 50 rats whose urine output was measured in each case at various times postoperatively. It will be noted that in the rat there is no "latent phase" of low fluid exchange such as is seen in the cat and the dog after denervation of the pars nervosa. By approximately 8 weeks after operation most of the rats were excreting urine within the range of the normal animals. Some rats were excreting at normal rates 6 weeks after operation; but others were still excreting above the normal rate for as long as 15 weeks postoperatively. This same slow recovery has also been observed by Dodds, Noble and Williams.[4] Three rats have been observed for 16 months postoperatively. At the end of this period, they were excreting at a rate of 4-6 cc per day—well within the normal range.

Histologic examination of the contents of the sella turcica of these recovered animals showed normal anterior lobe tissue (also, the females had normal estrual cycles). But invariably there was no remnant of pars intermedia. In the rat this structure surrounds the pars nervosa on all sides except the dorsal; the two structures are so closely applied to each other that it is impossible to pull them apart by traction with aspirators such as are employed in the operation. Therefore, since the surgical technic evidently removes entirely the pars intermedia and since the pars nervosa follows the intermedia in this operation, we feel certain that in the original operation all of the pars nervosa was removed except the few cells left around the attachment of the stalk. However, the histologic examination of the tissues left in the sella turcica of these recovered animals revealed the presence of large quantities of pars nervosa tissue. In some cases it was normal in both appearance and in gross quantity. It had, presumably, regenerated.

Fisher, Ingram and Ranson[5] (*cf.* also Gersh[6]) have emphasized that tissue resembling pars nervosa can be found in the infundibulum and hypothalamus and that all of this tissue must be removed to produce diabetes insipidus in cats. Fig. 1 shows that in the rat diabetes insipidus can be produced by removal of the tissue in the sella turcica only. Its transience, however, we feel to be due to incomplete removal with regeneration from the fragments of infundibular tissue left in the sella at operation.

The "transience" of our diabetes insipidus was far different from

[4] Dodds, E. C., Noble, R. L., and Williams, P. C., *J. Physiol.*, 1937, **91**, 202.

[5] Fisher, C., Ingram, W. R., and Ranson, S. W., "Diabetes Insipidus, etc.," Ann Arbor, Mich. 1938.

[6] Gersh, I., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 395.

that reported in dogs and monkeys by Keller, Noble and Hamilton[7] and by Mahoney and Sheehan.[8] Whereas their transient diureses are light and relatively short in duration (approximately 3 weeks), the diuresis in the rat was great (averaging 200 cc per kilo) and long in duration (8 weeks).

Although recovery can be shown in rats, no such phenomenon has been observed in cats after lesions to the supraoptico-hypophyseal tracts which causes a functional insufficiency of the pars nervosa.[5] The Ranson group have observed no recovery in one cat for 9 months after operation and in other animals for 5 months. Several explanations of this difference between their experiments and ours are possible. The most plausible is that mentioned above, *viz.*, incomplete removal of the tissues secreting antidiuretic hormone. That a permanent diabetes insipidus can be produced in the rat has been shown by Richter[9] with a technic entailing the transection of the pituitary stalk.

As has been previously reported,[10] giving these diuretic rats NaCl in their drinking solutions prevented the spontaneous recovery from posthypophysectomy that we have observed in the rat and, in fact, aggravated the diabetes insipidus considerably. It was also found that this aggravation could be prevented by giving $CaCl_2$ along with the saline solutions. Parathormone, however, was found to have no effect.

Studies of the basal metabolic rate in both operative diabetes insipidus and in salt-aggravated diabetes insipidus showed that this constant was not elevated, no matter how great the fluid exchange. It was also found that the presence of the thyroid was not necessary for the continuation of a salt-aggravated diabetes insipidus.

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7 Keller, A. D., Noble, W., and Hamilton, J. W., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 794.

8 Mahoney, W., and Sheehan, D., *Am. J. Physiol.*, 1935, **112**, 250.

9 Richter, C. P., The Pituitary Gland. Baltimore, Md., 1938.

10 Swann, H. G., and Penner, B. J., *Am. J. Physiol.*, 1938, **123**, 199.

10167

Cultivation of *Trichomonas foetus* in the Chick Embryo.*

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At the suggestion of Doctor A. L. Tatum, attempts were made to cultivate a bacteria-free strain of *Trichomonas foetus* upon the chorio-allantoic membrane of the chick embryo according to the window technic devised for the cultivation of viruses by Goodpasture.[1] These attempts were unsuccessful. However, by modifying the original technic, it was found that the living chick embryo could serve as a favorable medium for the cultivation of *Trichomonas foetus*.

The strain of *Trichomonas foetus* used in the experiment was the strain H of Glaser and Coria,[2] kindly sent to this laboratory by R. W. Glaser in November, 1935. Since that time it has been kept on Locke-egg-blood medium. Within 3 months after its arrival the strain was injected serially into the peritoneal cavities of 2 guinea pigs. It was recovered from the uterus of the first pig and freed from associated bacteria by the migration technic of Glaser and Coria. Like Rees,[3] the writer found that *Trichomonas foetus* migrates down more readily than up. The strain was recovered from the peritoneal cavity of the second pig in pure culture. This particular strain is now known in this laboratory as HGP2.

The inoculations of the chick embryos were performed in this manner: The chorio-allantoic membrane of an 11- or 12-day-old chick embryo was exposed. Sterile air was forced into the allantoic cavity through an artificially produced opening to make the membrane adhere to the edges of the shell opening. For the first inoculation, about one cc of a 4-day-old culture in L.E.B. medium was introduced into the allantoic cavity with a glass pipette. For subsequent inoculations, about one cc of allantoic fluid, withdrawn by means of a glass pipette from the allantoic cavity of an embryo inoculated 3 or 4 days previously, was used. Only those allantoic fluids were selected for injection which were found by direct microscopic examination to be teeming with living trichomonads, and by

* This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

1 Goodpasture, E. W., and Buddingh, G. J., *Am. J. Hyg.*, 1935, **21**, 319.

2 Glaser, R. W., and Coria, N. A., *Am. J. Hyg.*, 1935, **22**, 221.

3 Rees, C. W., *Am. J. Hyg.*, 1937, **26**, 283.

examination of smears stained by the Gram method to be free from bacteria.

The primary purpose of the experiment was to maintain the trichomonads in the chick embryo. Because of inadequate facilities, no infected embryos were kept for more than 3 or 4 days after being inoculated. During this 3- or 4-day interval, the total mortality of all embryos was 23%. No experiments have been performed to determine the effect of the trichomonads on the chick embryo. However, two observations indicate that, in the experiment described, no increase in pathogenicity of strain HGP2 occurred. First, there was no progressive increase in mortality of the embryos. A high mortality which occurred suddenly in the thirteenth and fourteenth sets of embryos was probably due to difficulties encountered with the incubator during that time. Secondly, embryos which were inoculated with material from dead embryos showed no significantly higher mortality than those inoculated on the same day with material from live embryos. The genealogy of some of the trichomonads could be traced through 14 embryos alive at the time of inoculation and at the time of removal of allantoic fluid.

Conclusion. *Trichomonas foetus* has been grown successfully in chick embryos through 14 generations (and probably could be continued indefinitely) when the parasites are inoculated beneath the chorio-allantoic membrane as described above.

10168

Further Studies on the Regeneration of the Aqueous in Man.

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If the aqueous of a human eye is aspirated through a fine hypodermic needle, introduced into the anterior chamber through the periphery of the cornea, several reactive processes are set up within the eye.* The formation of new intraocular fluid and characteristic reactive fluctuations of the intraocular pressure are probably the most important of these processes. We reported[1] the results of ex-

*This procedure is hereafter referred to as anterior chamber puncture (ACP).

1 Kronfeld, P. C., and Lin, C. K., *Transactions Amer. Acad. Ophth. and Otolar.*, 1937.

periments which were undertaken to determine the rate of formation of new intraocular fluid. The anterior chambers of human eyes which, from the viewpoint of the clinician, appeared to be nearly normal were emptied completely by ACP and the amounts of fluid thus obtained measured. One hour later this procedure was repeated whereby the amounts of fluid regenerated in one hour were obtained. These data were interpreted to indicate independence of the rate of fluid regeneration from the original chamber volume. "According to these data, the regeneration of the aqueous takes place at the same absolute rate in all the eyes represented in the table, the result being that an originally deeper chamber." This interpretation was corroborated by the results of experiments in which the intraocular tension was followed after ACP. The so-called restoration time (the interval between the ACP and the time at which the original level of tension is reached again) was found to be directly dependent upon the original volume of the chamber, longer restoration times being characteristic of eyes which had deeper anterior chambers originally and *vice versa*.

In the same communication it was also reported that the intensity of some of the other reactive changes which follow ACP appears to be proportional to the intensity of the eliciting stimulus, that is, to the amount of fluid withdrawn or, in other words, to the amount of fluid originally contained in the anterior chamber. It was difficult to understand that this proportionality should only apply to some of the reactive changes and not also to the rate of fluid regeneration. We have, therefore, continued our experiments and determined, on a number of fairly normal eyes, the amount of fluid regenerated within the first 30 minutes after complete emptying of the anterior chamber. The punctures were made with 23 gauge needles under local anesthesia which consisted of 4 instillations of 2 drops each of 1% pantocaine given at intervals of 3 minutes, plus the direct application of the same solution with cotton applicators to the upper and lower limbus at which places the eyeball was held with 2 fixation forceps during the puncture.

In none of these cases did the ACP produce any lasting untoward effects with regard to appearance or function of the eyes which were subjected to this procedure. In some of the cases of retrobulbar neuritis or optic atrophy temporary or lasting improvement of the vision was noted after the ACP. ACP may, therefore, be considered a therapeutic procedure in these cases.

The volumes of aqueous obtained by the first (v_1) and the second (v_2) ACP are presented in Table I and Graph 1. Before interpreting these data the principal sources of error involved in such

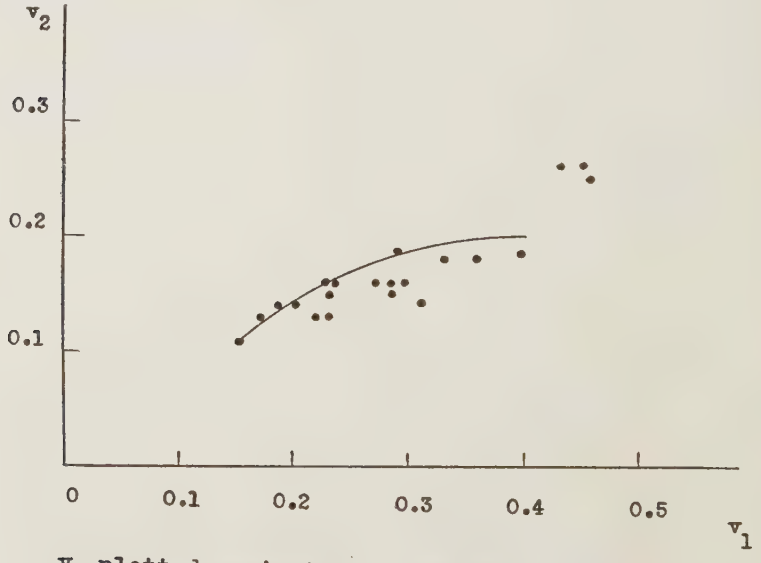
TABLE I.

No. of patient	Age	Vol. of aqueous in cc obtained at 1st ACP (V_1)	2nd ACP (V_2)	Min. between 1st and 2nd ACP	Clinical condition
61408	37				Concomitant convergent strabismus of the right eye. Left vision: with + 2.00 sph = 6/6. Right vision: with —8.00 sph with —1.25 cyl × 150 = 6/10
Left Eye		.172	.130	30	
Right "		.235	.160	30	
61342	14				Mild chronic retrobulbar neuritis of both eyes, probably due to dietary (vitamin) deficiency. Left vision: with —4.00 sph = 6/10. Right vision: with —4.00 sph with —1.00 cyl × 30 = 6/10
Left Eye		.430	.260	31	
Right "		.455	.250	31	
58469	19				Concomitant divergent strabismus of the right eye. Right vision: with —0.75 sph with +1.50 cyl × 150 = 6/6. Left vision: with +1.25 sph = 6/5-2
Right Eye		.288	.185	30	
60810	31				Polyneuritis and retrobulbar neuritis of both eyes, due to dietary (vitamin) deficiency. Left vision: 6/10. Right vision: with —0.25 sph with +0.50 cyl × 105 = 6/10
Left Eye		.185	.140	30	
Right "		.200	.140	30	
383204	22				Retrobulbar neuritis of both eyes, cause unknown. Left vission = 6/15, Jaeger 4
Left Eye		.330	.180	29	
60813	24				Polyneuritis (without retrobulbar neuritis), due to dietary (vitamin) deficiency. Right vision: with —0.75 sph = 6/10
Right Eye		.220	.130	31	
56184	15				Concomitant convergent strabismus, alternating. Right vision: with —0.25 sph with +1.25 cyl × 60 = 6/10
Right Eye		.286	.150	30	
61821	25				Polyneuritis (without retrobulbar neuritis), due to dietary (vitamin deficiency), slight degree of vitamin A deficiency. Left vision: with —0.50 sph = 6/10. Right vision: with —0.50 sph = 6/15 + 3
Left Eye		.355	.178	31	
Right "		.394	.186	31	
57957	21				Chronic retrobulbar neuritis of both eyes, cause unknown, partial optic atrophy of both eyes. Left vision = 6/20, Jaeger 2. Right vision = 6/20, Jaeger 2
Left Eye		.237	.160	33	
Right "		.230	.150	30	
61470	24				Polyneuritis (without retrobulbar neuritis), due to dietary (vitamin) deficiency. Left vision = 6/10 (small central corneal scar)
Left Eye		.230	.130	30	

TABLE I (Continued).

No. of patient	Age	Vol. of aqueous in cc obtained at 1st ACP (V_1)	2nd ACP (V_2)	Min. between 1st and 2nd ACP	Clinical condition
389674	18				Retrobulbar neuritis of both eyes, cause unknown, partial optic atrophy of both eyes. Left vision = counting of fingers at 3 meters. Right vision = 3/20
Left Eye					
3-25-38		.296	.160	30	
4-8-38		.280	.160	30	
Right Eye		.310	.139	30	
Left Eye	45	.150	.113	30	Mild trachoma, corneal scars, presbyopia. Left vision = 6/20, with +2.50 sph Jaeger 5
364304	20				Healed retrobulbar neuritis of both eyes, cause unknown. Left vision =6/6, Jaeger 1
Left Eye		.268	.160	30	
363813	16				Myopia. Right vision: with —.400 sph = 6/20, Jaeger 1
Right Eye		.450	.258	30	

experiments should be considered. As stated in our previous communication, the determination of the volume of chamber fluid cannot be done with great accuracy because it is difficult to aspirate the last trace of fluid. The amount of aqueous left in the anterior chamber in the experiments reported here probably did not exceed 0.01 cc. Another possible source of error is loss of intraocular fluid by leakage during the interval between the two ACP. As a rule, the



v_2 plotted against v_1. Interval between the two aspirations 30 minutes.

Graph I.

track made by the first ACP becomes watertight immediately. Occasionally, however, the canal stays open for a period of hours or even days; the eye is said to be "fistulating". In the experiments reported here special attention was paid to the possibility of fistulation, but no signs of it could be noticed. Both of these sources of error tend to lower the results. The curve of Graph 1 is, therefore, based chiefly upon the higher and not upon the lower readings. Errors may also arise from heterogeneity of the material with regard to the characters which are being studied, that is, the depth or volume of the anterior chamber and the capacity of the fluid-producing apparatus. Unfortunately, the criteria of homogeneity or of heterogeneity with reference to these characters are not definitely known. Only the cases 61342 and 363813 of Table I with their unusually deep chambers and unusually high rates of regeneration seem to be definitely of a different type than the other cases.

The data contained in Table I and Graph 1 show that there is a relationship between the absolute amount of fluid which is regenerated within 30 minutes after complete ACP (v_2) and the original volume of the anterior chamber (v_1), v_2 showing a definite tendency to be higher in eyes with high v_1 and *vice versa*. Our findings are not regular enough to allow expression in mathematical terms. The relation between v_2 and v_1 does, however, not seem to be that of direct proportionality. Whatever the actual mathematical relationship between v_2 and v_1 may be, it is justifiable to state that the rate of fluid regeneration is, like some of the other reactive processes which follow ACP, dependent upon the original chamber volume. This chamber volume may, in turn, be considered a measure of the magnitude of the stimulus which the ACP constitutes for the eye.

The question may be asked why the data presented in the previous communication did not bring out this relationship. The answer is that the depths of the anterior chambers of the eyes in this first series (the independent variable) did not vary sufficiently, most of these eyes having anterior chambers of average depth. A material of this composition while it is almost ideally homogeneous can not be expected to bring out a regularity in the variation of the dependent variable (v_2).

10169

Platelet Studies in Menstruation and Hemophilia: Total and Differential Counts, Disintegration Rates and Lipid Distributions.

Pearl Lee and Betty Nims Erickson. (Introduced by I. G. Macy.)

From the Children's Hospital of Michigan and Research Laboratory, Children's Fund of Michigan, Detroit.

Few observations of any kind have been made on the platelets of menstruating women although Genell[1] called attention to the variations in platelet counts during menstruation. According to Birch[2, 3] partial control of the hemorrhages of hemophilia may be obtained by treatment with ovarian extract. She has pointed out that it is possible that the female carrier is protected from bleeding by her sex hormones. This possibility suggested that a comparative study of the platelets in hemophilia and in menstruation might reveal interesting facts. To this end total and differential counts, disintegration rates, and detailed lipid studies were made upon platelet preparations from the blood of normal women, between and during menstruations, and hemophilic children.

The technic of Olef[4] for platelet counting and determination of disintegration rate[5] * was chosen for these studies. In this indirect method, to prevent clumping and disintegration, a drop of sodium metaphosphate is placed on the site of the finger puncture so that the blood is mixed directly with the diluting fluid and does not contact the skin. After transferring to a paraffine cup, fresh preparations are made from the mixture for counting. A simultaneous erythrocyte count is also necessary to evaluate the absolute number of platelets. The platelets are differentiated into 3 groups according to size, using the red cell as a standard: Group I is one-fourth the size of the red cell, Group II is one-third, and Group III is one-half.

Observations were made on 10 normal women when not menstruating and 9 hemophilic children. Five of the women were studied

1 Genell, S., *J. Obst. and Gynec.*, Brit. Empire, 1936, **43**, 1124.

2 Birch, C. L., Proc. Soc. Exp. Biol. and Med., 1930-31, **28**, 752.

3 Birch, C. L., *J. A. M. A.*, 1932, **99**, 1566.

4 Olef, I., *J. Lab. and Clin. Med.*, 1935, **20**, 416.

5 Olef, I., *J. Lab. and Clin. Med.*, 1936, **22**, 128.

* Olef's technic for disintegration rate was modified slightly by placing the paraffine cup (containing the blood) in the incubator at 37°C for 6 hours, instead of in the refrigerator for 8 hours.

TABLE I.
Total and Differential Platelet Counts and Disintegration Rates.

	Untreated hemophiliacs		Normal Women			
			Non-Menstruating		Menstruating	
	Avg of 7 determinations on 4 subjects*	One subject	Avg of 5 determinations on 4 subjects	One subject	Avg of 7 determinations on 5 subjects	One subject
Clotting time		2 hr		11 min		20 min
Total platelet count in thousands:						
Initial		450		828		330
Incubated 6 hr		520		494		374
Disintegration rate	+5%	+16%	—41%	—40%	+2%	+13%
Differential platelet count:						
Group I:						
Initial		329		248		122
Incubated 6 hr		140		213		104
Increase or decrease	—25%	—57%	—30%	—14%	+9%	—15%
Group II:						
Initial		117		580		208
Incubated 6 hr		380		281		225
Increase or decrease	+62%	+225%	—34%	—52%	—3%	+8%
Group III:						
Initial		4.5		—		0
Incubated 6 hr		—		—		45

*Five of the hemophiliacs are not included in this tabulation because they had received treatment. They will be discussed in a later paper.

during menstruation. Sixty-five total and differential platelet counts and 20 disintegration rates were determined. Complete data are given in Table I upon one hemophilic child, one non-menstruating woman, and one menstruating woman, and the average percentages for the groups.

It may be seen that the non-menstruating women have a decided decrease in both Groups I and II while the hemophiliacs show a decrease in Group I and a marked increase in Group II. The disintegration rate figures obtained during menstruation fall between the normal and the hemophiliacs. The most striking similarity of the platelets of hemophilia and menstruation is found in the slower (+) disintegration rate.

The increase in Group II found in hemophilia may be due to the initial swelling that the platelets have undergone in the first stage of the process of normal disintegration. Ferguson,[6] in studying disintegration of normal platelets by means of the dark field, has observed that the first change is a swelling into a "spherule". Suspension of the process at this stage would explain an increase in the larger forms and a decrease in the smaller.

Since cephalin has been shown to play an important part in blood clotting, detailed lipid studies were made on the platelets of menstruating and non-menstruating women. Platelet samples were collected from the blood of 5 normal women during the first days of menstruation and were pooled for chemical analyses.† The lipid composition, together with comparative data on a pooled platelet sample collected from the blood of 5 normal women during the intermenstrual period,[7] is presented in Table II.

TABLE II.
Lipid Composition of Human Platelets Between and During Menstruations.

	Inter-Menstruation Avg for 5 subjects		Menstruation Avg for 5 subjects	
	Dry weight of platelets %	Total lipid %	Dry weight of platelets %	Total lipid %
Protein	69	—	73	—
Total Lipid	16	—	17	—
Phospholipid	12	75	14	82
Free Cholesterol	2	13	2	12
Cholesterol Esters	2	12	1	6
Neutral Fat	0	0	0	0
Cephalin (% of total phospholipid)		68		85

[6] Ferguson, John H., *Am. J. Physiol.*, 1934, **108**, 670.

† The detailed procedure for the preparation of platelet samples and methods for chemical analyses are given in a preceding report.[7]

[7] Erickson, Betty N., Lee, Pearl, Williams, H. H., and Avrin, I., *J. Clin. Invest.* In press.

In contrast to their altered physical behavior, the blood platelets during menstruation exhibit no deficiency in their lipid composition. In fact, a greater proportion of the clot-aiding type of phospholipid, cephalin, appears to be present. No anomaly could be detected in the lipid composition of platelets from the blood of hemophiliacs; a discussion of other factors which must be investigated in relation to the physical behavior of blood platelets has been given.[7]

Summary. A definitely delayed platelet disintegration rate in menstruation is demonstrated by these studies. Hemophilia also shows similar changes. Lipid analysis of the platelets demonstrates no deficiency of cephalin in either menstruation or hemophilia.

10170

Time of Death of Lethal Homozygotes in the *T* (Brachyury) Series of the Mouse.*

S. GLUECKSOHN-SCHOENHEIMER. (Introduced by L. C. Dunn.)

From the Department of Zoology, Columbia University.

Dunn[1] reported the third lethal that was found in the *T* (Brachy) series in the house mouse. This lethal (t^1) was determined by Dunn in a tailless line, which had been isolated by Dobrovolskaia-Zavadskaia and Kobozieff.[2] Tailless mice of this line (29) are Tt^1. Results of matings of tailless mice of line 29 and tests of their descendants showed that both homozygous combinations TT and t^1t^1 are lethal, but the combination Tt^1 is viable.

In order to learn more about the lethal embryos t^1t^1, uteri of pregnant females were examined in different stages. The females used were all heterozygous normal-tailed mice ($+t^1$) and they were mated to heterozygous normal-tailed males ($+t^1$).

Table I shows the results of dissections of pregnant females at the age of 7, 8, 9, 10, and 11 days after fertilization. A total of 40 litters were dissected out, yielding 294 embryos. Of these, 275 were normal and 19 abnormal. Of the abnormal embryos, 13 were resorbed and 6 showed different kinds of abnormalities, but were not resorbed. If the t^1t^1 homozygotes died after implantation,

* This research was aided by a grant from the Josiah Macy, Jr., Foundation.

1 Dunn, L. C., *Proc. Nat. Ac. Sc.*, 1937, **23**, 474.

2 Dobrovolskaia-Zavadskaia, N., and Kobozieff, N., *C. R. Soc. Biol.*, 1932, **110**, 782.

TABLE I.

Exp.	Age of embryos	No. of litters	No. of embryos	Normals	Resorbed
	days				
1	7	8	51	49	2
2	8	13	101	97	4 (2?)†
3	9	6	40	38	2
4	10	8	59	54	5
5	11	5	43	37	6 (4?)
Total		40	294	275	19 (6?)

† Figures in parentheses represent abnormal, but not resorbed embryos.

25% of the implanted embryos would be expected to be of the lethal type. Since the number of resorbed embryos found at dissection is far below expectation, it has to be assumed that death of the homozygous lethal takes place before implantation, and that the death of the resorbed and abnormal embryos found was due to accidental causes. It is highly improbable that death of the t^1t^1 takes place after the 11th day. All embryos examined at 11 days were entirely normal and no indications were found for later death of t^1t^1.

The litter size in the matings is another factor indicating that death of t^1t^1 occurs before implantation. The average litter size in matings of heterozygous normal-tailed mice of the tailless A line *inter se*—where all embryos are implanted—was found to be 9.8 (taken from 14 litters), the average litter size in matings of $+t^1$ normals *inter se* was 7.4 (taken from 40 litters). If the t^1t^1 homozygotes died before implantation one would expect the number of implanted embryos to be 25% smaller than it would be if *all* embryos were implanted. The figures found correspond very well to expectation.

The study of litters from matings of heterozygous normal-tailed mice in the A line *inter se* ($+t^0 \times +t^0$) confirmed Chesley's observation that death of the homozygous lethal in the A line occurs after implantation (Chesley and Dunn).[3] Of 170 embryos at ages from 6-8 days that were examined in dissections and histologically so far, 115 were found to be normal, the rest abnormal or resorbed. Death of t^0t^0 apparently takes place on the 6th day of development. The details of this investigation on the t^0-homozygote will be described in a later publication.

This investigation was undertaken at the suggestion of Dr. L. C. Dunn, whom I should like to thank for his help during the course of the work.

3 Chesley, P., and Dunn, L. C., *Genetics*, 1936, **21**, 525.

10171

Comparison of the Beats in Three and Four Chambered Hearts.

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From the Departments of Zoology and Physiology, University of Illinois, Urbana.*

A careful observation of the beating hearts of the cat and turtle shows a marked dissimilarity in the manner of contraction. The cat heart appears to remain relatively constant in length, but expands and contracts laterally with each beat, while the turtle heart may be seen to contract uniformly in all directions, as a ball of muscle.

In our attempt to examine this phenomenon in a quantitative way, we used the method outlined by Takeuchi[1] who used cinematographs to examine volume changes of the heart during anoxemia. In our experiments a 16 mm Filmo motion picture camera was used to photograph the beating hearts, exposed *in situ*, at speeds of either 8 or 16 pictures a second. Light was obtained from Eastman photoflood lamps.

After the pictures had been taken, each frame was studied under the binocular microscope, using an ocular micrometer which gave a value of one ocular unit equal to ½ mm. By means of this, measurements were made of the greatest width and length of the ventricular picture on successive frames for 5 or 6 beats. The amount of change could be plotted graphically against time (in exposures) to form curves similar to the kymograph cardiogram. From these curves the actual dimensions of the hearts during the cycle could be determined for any point on the cycle. Differences between systolic and diastolic sizes along the 2 axes studied were then translated into percentages of the maximum dimensions.

From the above data it may be seen that the ratio between the contraction of the width and length in the heart of the turtle is much nearer 1.0 than is the ratio of the cat's heart. In most cases the turtle ventricle contracts longitudinally even more than it contracts transversely, thus giving ratios of less than 1.0, in distinct contrast to the condition in the cat. Pictures made of the heart beat in the undivided ventricle of the fish and the double ventricle of the bird show results which agree with the ratios of the turtle and cat respectively. Although no definite statements can yet be made, varia-

* Contribution from the Zoological Laboratory of the University of Illinois, No. 522.

1 Takeuchi, K., *J. Physiol.*, 1925, **60**, 209.

TABLE I.

Animal	No. of observations	% contraction in width	% contraction in length	Width / Length Ratio
Turtle	1	22.4	25.2	0.888
	2	24.7	29.3	0.843
	3	12.3	10.3	1.194*
	4	30.8	30.0	1.025
	5	30.7	37.5	0.818
	6	21.1	20.0	1.055
	7	20.1	20.3	0.990
	8	10.7	12.5	0.856
	9	16.15	20.7	0.780
	10	15.3	19.6	0.780
Catfish	1	23.8	18.9	1.26
Cat	1	7.1	1.8	3.95
	2	6.6	1.5	4.30
	3	7.0	1.7	4.11
	4	5.0	1.56	3.12
	5	11.96	4.41	2.72
	6	13.33	4.05	3.30
Chicken	1	7.8	3.5	2.23

*Isolated heart.

tions of the ratio in different experiments may be correlated with the rate of beat and intraventricular pressure.

These figures indicate that the contraction in the single ventricles of the turtle and fish is the result of the nearly equal participation of both longitudinal and transverse muscle fibers, while contraction in the double ventricles of the cat and bird is largely the result of the transverse muscles, the longitudinal contraction being relatively small.

For a possible explanation of this difference one might consider the intraventricular septum as a buttress against any movement in the direction in which it lies, but affording no resistance to movements in the lateral direction. On the other hand, since the septum is absent in the hearts of the turtle and fish, there is nothing to prevent the muscle from contracting more or less equally in all directions and giving the contracting ventricle a ball-like appearance. These differences between the length and the width in the 3- and 4-chambered hearts may also be related to the structural arrangement of the inner and outer muscle layers.

10172 P

A Method for Quantitative Estimation of Pigment in Gallstones.*

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None of the methods known for quantitative estimation of bile pigment has been found satisfactory for gallstone pigment estimations. van den Bergh's method[1] is unsatisfactory, as the unoxidized fractions of pigments are not included; the method of Schmidt and Jones[2] is objectionable for similar reasons; Peterman and Cooley[3] carried oxidation with peroxide to a blue end-point and used a light filter when making the colorimetric reading.

The method offered here uses perchloric acid for oxidation, as the steps are better controlled and the end-products are more stable. A light filter is not necessary with this method. Bile pigments are extracted from weighed amounts (10-20 mg) of dry, well powdered stones, by refluxing with 5 to 8 cc of a mixture of equal parts of chloroform, ethyl alcohol, and glacial acetic acid, until the solvent drops colorless from the tip of the filter cone holding the powdered stone. Previous to this, cholesterol, and calcium and phosphorus are removed by washing first with warm ether and then with hydrochloric acid. Excess temperature and prolonged extraction are avoided to diminish the oxidation as far as possible. The extract is cooled to room temperature and made to a known volume, using the triple mixture. Aliquots (usually 1-2 cc) of this extract, as the intensity of the solution indicates, are measured into small test tubes and made up to a volume of 3 cc with 95% alcohol. At least one cc of the alcohol should be used.

Oxidation to a blue color is accomplished by the addition of 0.8 cc of 72% perchloric acid. Colorimetric comparison is made after 10 minutes against an aliquot from a known strength of bilirubin solution in the triple mixture, which has been oxidized at the same time and in the same manner. A standard from a 3-5 mg %

* This work was done in part under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

1 van den Bergh, Hymans A. A., *Press Med.*, 1921, **29**, 441.

2 Schmidt, C. R., Jones, K. K., and Ivy, A. C., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 17.

3 Peterman, E. A., and Cooley, T. B., *J. Lab. and Clin. Med.*, 1934, **19**, 723, 743.

solution of Eastman's bilirubin has been found satisfactory for the depth of readable color.

Bilirubin dissolved in this triple mixture, when exposed to heat, is recovered within the range of experimental error; when previously treated with ether and hydrochloric acid and then exposed to heat, about 70% of the bilirubin is recovered. The loss is chiefly due to the differences in surface tension between the bilirubin and dilute hydrochloric acid. Gallstones do not show this difference, so we feel that little mechanical loss occurs on treating them. Chemical factors may also play a part in the recovery. Figures obtained by this method[4] do not always check within a close range, yet they give a fair idea of the total amount of pigment in stones.

10173

Anticatalase Activity of Sulfanilamide and Related Compounds. I. Effect of Ultraviolet Irradiation.

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The production of a violet color by irradiation of dilute solutions of sulfanilamide with ultraviolet light has been reported by Ottenberg and Fox.[1] They suggested that this colored substance, possibly produced by oxidative changes in the body, may be responsible for the cyanosis frequently observed in patients under treatment. Whether or not the colored derivative was superior to sulfanilamide in bactericidal power was not determined.

In work to be published[2, 3] it has been suggested, as a possible explanation of the retardation of growth of pneumococci by sulfanilamide, that the bacteriostatic agent involved may be—not sulfanilamide itself—but hydrogen peroxide. The latter substance was presumed to accumulate in the immediate locality of the invading coccus, following oxidation by the coccus of sufficient absorbed sulfanilamide to produce inhibition of catalase as rapidly as the latter principle enters the reaction zone. The oxidation product may be

4 Phemister, D. B., Aronsohn, H. G., and Pepinsky, R., in press.

1 Ottenberg, R., and Fox, C. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 479.

2 Locke, A., Main, E. R., and Mellon, R. R., *J. Immunol.*, in press.

3 Mellon, R. R., *Mod. Hosp.*, 1938, **51**, 53.

similar to that produced by irradiation. Qualitative tests showed that irradiated sulfanilamide had a marked inhibitory effect on the activity of serum catalase. This report presents further data, showing that non-irradiated sulfanilamide also has appreciable anticatalase activity, which is increased upon irradiation. The observations of Ottenberg and Fox regarding color production have been extended to a number of related compounds, active and inactive therapeutically, and it has been shown that, in many of these, anticatalase activity either appeared, or was enhanced, after irradiation.

Solutions were irradiated for one minute in thin layers at a distance of 3 inches from a Westinghouse Sterilamp. The results of Ottenberg and Fox, showing that the color produced varied with the concentration of solution used, were confirmed. The color was first detectable at a concentration of about 0.2 mg %. Up to a concentration of 2 mg % the intensity of color was directly proportional to the concentration. Above this concentration the amount of color became increasingly less and at concentrations greater than 16 mg % the color produced was yellow to brownish red. The violet color faded rapidly to a brownish pink on standing. This instability was apparently related to the production of acid during irradiation since the addition of a trace of bicarbonate solution at the time of irradiation stabilized the violet color so that it was maintained for several days. A pink color was produced when the solution irradiated was slightly acid in reaction or when acid was added to the violet-colored solutions.

Attempts to produce a comparable color in sulfanilamide solutions by oxidation with the commonly used oxidizing agents were unsuccessful. The color of irradiated sulfanilamide solution was destroyed by treatment with hydroquinone and could not be restored by hydrogen peroxide.

Substances which produce inactivation of heavy metals[4] modified the effect of ultraviolet irradiation. The addition of diethyl dithiocarbamate, potassium cyanide, or potassium thiocyanate, compounds which act primarily on copper, completely inhibited production of color in irradiated solutions. Sodium pyrophosphate, which inhibits iron, did not interfere with color production. Sodium fluoride, a still more effective inhibitor of iron, caused intensification of color.

The effect of irradiation upon solutions of 17 compounds,* related to sulfanilamide in structure, is summarized in Table I. In 6

[4] Locke, A., and Main, E. R., *J. Infect. Dis.*, 1931, **48**, 419.

* Synthesized and donated to us by the Monsanto Chemical Company, St. Louis, Missouri.

of these, definite colors were produced. The color produced by compounds 11 and 16 resembled that given by sulfanilamide. Compounds 7, 8, and 13 yielded a sky-blue color. No color was produced in solutions of compounds which have substituents in the *p*-amino group or aryl substituents in, or replacing the sulfamido group. In general, color was produced only by those compounds in which the *p*-amino group was free and in which the sulfamido group was either free, replaced by an alkyl group or partly substituted by an alkyl. Thus, the color-producing compounds fell into two groups: (a) Compounds 11, 16 and 18, in which the sulfamido group was free or partly alkylated, produced a violet color, unstable in the presence of acid. (b) Compounds 7, 8, and 13, in which the sulfamido group was replaced by an alkyl group, gave a blue color, unstable in alkaline and neutral solution.

Estimations of anticatalase activity of irradiated and non-irra-

TABLE I.

Color Production by Ultraviolet Irradiation of Solutions of Sulfanilamide and Related Compounds and Anticatalase Activity Before and After Irradiation.

Compound	Color produced	Anticatalase activity		
		Before irrad.	After irrad.	Conc. of soln. mg %
1. Phenyl sulfanilate	None	10	10	2
2. Benzyl p-aminophenylsulfone	"	10	10	2
3. N,N'-diacetylsulfanilamide	"	*	*	*
4. N-(p-aminobenzenesulfonyl)-benzamide	"	17	10	2
5. p-(n-hexylamino)-benzene sulfonamide	"	*	*	*
6. N-(p-aminobenzenesulfonyl)-imino diacetic acid	Trace?	0	57	8
7. Methyl-p-aminophenylsulfone	Blue	0	57	8
8. n-amyl-p- "	"	0	43	8
9. p-acetyl-amino-sulfanilamide	None	0	0	8
10. p-benzyl-amino-benzenesulfonamide	"	0	23	2
11. N,N'-di(p-aminosulfonyl)-ethylene-diamine	Red violet	10	70	2
12. p-(n-amylamino)-benzene-sulfonamide	Trace?	10	53	2
13. β-hydroxyethyl-p-aminophenyl-sulfone	Blue	11	67	8
14. 4,4'-diamino-benzenesulfon-anilide	None	61	58	8
15. 4,4'-di-(acetylamino)-diphenyl-sulfone	"	*	*	*
16. N-(p-aminobenzenesulfonyl)-aminoacetic acid	Red violet	12	69	8
17. Sulfanilic acid	None	9	0	8
18. p-aminobenzenesulfonamide	Violet	17	70	2
18. "	"	17	70	8

* Too insoluble for test.

diated solutions of sulfanilamide showed that a marked increase in such activity appeared simultaneously with the development of color. A 10% dilution of fresh rabbit serum in water was used as a source of catalase and its activity was determined by titrating the unchanged hydrogen peroxide with standard permanganate solution. For the determination of anticatalase activity, a solution of the compound to be tested was used as a serum diluent. After a reaction period which allowed decomposition of about 50% of the added peroxide, the mixture was acidified with *p*-toluene sulfonic acid and titrated immediately.

The amount of active catalase was calculated from the formula: $E = x^2/A$, where x is the amount of peroxide decomposed and A is the amount originally present. The time and temperature factors were considered to be constant. The anticatalase activity or percent suppression of catalase was then expressed as 100—(100 E/E′), where E′ is the total catalase value obtained with water. Values of 10 or less were not considered significant. The slight acidity produced in irradiated solutions did not interfere with the determinations. A series of 15 estimations of the anticatalase activity of 8 mg % solutions of sulfanilamide showed that comparable results could be obtained with different sera. The average activity of irradiated solutions was 83, that of non-irradiated, 27.

The effect of the time of irradiation, concentration and aging on the anticatalase activity of sulfanilamide is reported in Table II. With an 8 mg % solution, a maximum activity was reached after irradiation for 45 seconds or less. The activity of irradiated solutions increased with increasing concentration of sulfanilamide up to a concentration of 1-2 mg %, but no further increase in activity was observed at higher concentrations. Conversely, upon dilution

TABLE II.

Effect of Radiation Time, Concentration, and Aging on the Anticatalase Activity of Sulfanilamide Solutions.

Effect of radiation time (8 mg % soln.)		Effect of concentration at time of radiation		Effect of dilution after radiation		Effect of aging in radiated 8 mg % solutions	
Time, sec.	Anticat. activity	Conc., mg %	Anticat. activity	Conc., mg %	Anticat. activity	Days after radiation	Anticat. activity
0	25	8	76	8	87	0	89
5	42	4	73	4	80	1	93
15	67	2	73	2	80	2	73
45	88	1	76	1	73	4	57
120	88	0.5	64	0.5	67		
		0.2	56	0.25	41		
		0.05	36	0.10	37		
		0.025	0?				

of irradiated solutions, no decrease in activity occurred until a concentration of 1-2 mg % was reached. The anticatalase principle was found to be more stable than the substance responsible for the color. Thus solutions showed marked activity after standing for two days at room temperature, whereas the color faded within 2 hours. Although anticatalase activity develops simultaneously with color, the substances responsible for activity and for color are not identical.

Table I shows the relative anticatalase activities of the compounds listed. Compounds 3, 5, and 15 could not be tested because of their extreme insolubilities. Of the 9 compounds which had activities of 40 or more after irradiation, 6 produced definite color and 2 yielded traces of color. Compound 14 was exceptional. It gave no color on irradiation and had a high anticatalase activity both before and after irradiation, possibly due to its sensitiveness to air oxidation.

Three of the compounds listed, 4, 14, and 18, are known to possess therapeutic activity[5] in pneumococcal and some streptococcal infections. It is significant that they showed anticatalase activity without activation by ultraviolet light. A high anticatalase activity, developed as a consequence of irradiation, gave no information as to the therapeutic value of a compound.

Work is now in progress in which an attempt is being made to correlate the anticatalase activity of sulfanilamide with the retardation of growth in pneumococcus cultures.

Conclusions. The ability of sulfanilamide and structurally related compounds to develop color on ultraviolet irradiation appears to be dependent on the presence or absence of substituents in the *p*-amino and sulfamido groups. A study of the anticatalase properties of sulfanilamide and related compounds indicates a possible correlation between therapeutic activity and the intrinsic anticatalase activity associated with the non-irradiated compound. Neither color production nor the high anticatalase activity developed as a consequence of irradiation appear to be related to therapeutic effectiveness.

[5] Mellon, R. R., Gross, P., and Cooper, F. B., *Sulfanilamide Therapy of Bacterial Infections*, Charles C. Thomas, Springfield, Illinois, 1938.

10174

Visualization of Intrathoracic Vena Cava, Effect of Respiration on Diameter of the Vessel.

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The work of Franklin, Janker, Naegeli and others[1–6] has shown that the thoracic portion of the posterior vena cava undergoes changes in volume during respiration; that a lengthening, and therefore a decrease, in caliber occurs with each contraction of the diaphragm. These workers concluded that the blood flowing in the thoracic portion of the posterior vena cava is subjected to considerable churning. We are particularly interested in this question because of its important bearing on the accurate measurement of blood flow with the thermostromuhr. More nearly complete knowledge of the degree of changes in diameter of this vessel occurring during the respiratory cycle seemed imperative and, in the present report, our observations are recorded.

Four normal, well-trained dogs were selected for the investigation. With the animals under ether anesthesia and with the use of surgical technic, thorotrast (thorium dioxide) was injected directly into the wall of the vena cava as was done by Steggerda and Gianturco[7] in their studies on the colon. This method of rendering a blood vessel opaque to roentgen rays has the advantage of being more physiologic than the introduction of the material into the circulation, as was done by previous workers. It has the added advantage of being relatively permanent since the wall of the vena cava retains the thorotrast for an indefinite period and repeated roentgenograms can be taken as desired. The veins of dogs injected more than a year before seem just as opaque to roentgen rays as immediately after injection. The walls of the vena cava are so clearly defined that satisfactory measurements of changes in diameter can be made.

1 Franklin, K. J., *J. Physiol.*, 1933, **79**, 470.

2 Franklin, K. J., A monograph on veins, Springfield, Illinois, Charles C. Thomas, 1937, Chap. XIX, p. 236.

3 Franklin, K. J., and Janker, R., *J. Physiol.*, 1934, **81**, 434.

4 Franklin, K. J., and Janker, R., *J. Physiol.*, 1936, **86**, 264.

5 Franklin, K. J., and McLachlin, A. D., *J. Physiol.*, 1936, **87**, 87.

6 Naegeli, T., and Janker, R., *Deutsch. Z. f. Chir.*, 1931, **232**, 560.

7 Steggerda, F. R., and Gianturco, *Anat. Rec.*, 1937, **67**, 405.

The first roentgenograms were taken with the dogs lying on their sides (Fig. 1). In this position changes in diameter of the vena cava resulting from inspiration and expiration could not be detected except by actual measurement, which showed a slight decrease during inspiration. Besides the single roentgenograms, a series of pictures, one each second, was taken on roentgenographic film. By this means the size of the vena cava could be followed throughout the respiratory cycle. Later, the dogs were trained to stand in a Pavlov rack and roentgenograms were made both from the lateral and dorsoventral aspects.

In order to determine whether the method used to visualize the vena cava was capable of demonstrating changes in diameter 2 critical experiments were performed. The walls of the venae cavae of the dogs used in the experiments were injected with thorotrast as in the other experiments. Under amytal anesthesia, roentgenograms were taken during voluntary and artificial respiration. After laparotomy roentgenograms were taken while traction was applied to the stomach and liver, which exerted traction on the vena cava. Study of the roentgenograms disclosed that voluntary respiration

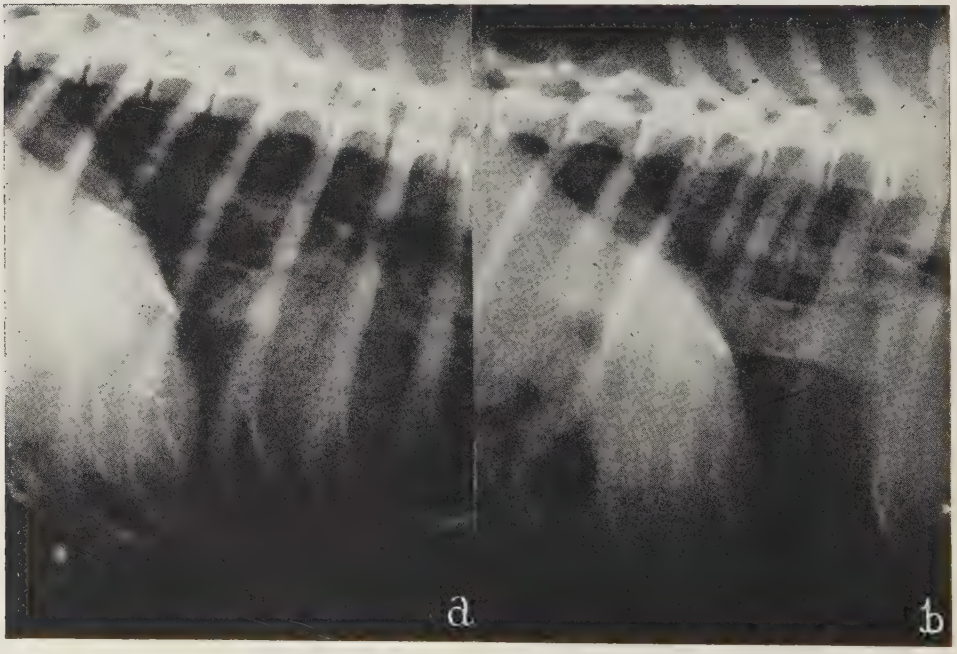

FIG. 1.

Lateral thoracic roentgenograms showing the intrathoracic portion of the inferior vena cava, *a*, during inspiration and *b*, during expiration. Dog lying on side.

caused slight or no changes in diameter while artificial respiration caused definite but small changes. This was probably owing to a greater volume of air being supplied than was inspired with normal respiration, artificial respiration resulting in a greater displacement of the diaphragm Very marked reduction in the diameter of the vena cava was produced by traction on the stomach and liver.

These relatively negative results in regard to changes in the diameter of the vena cava during voluntary respiration raised the question whether the position of the dog influenced the diameter of the vessel. Roentgenograms were taken of the vena cava while the dog was standing in a Pavlov rack. The horizontal diameter of the vessel could be recorded by a roentgenogram made in the dorsoventral direction. The vertical diameter could be recorded by a roentgenogram taken in the lateral direction. The dorsoventral exposure showed no significant difference between the diameter during inspiration and that during expiration. However, the vertical diameter was distinctly shortened during inspiration (Fig. 2). It is evident, therefore, that the position of the dog definitely affects the diameter of the vena cava during respiration. This may be attributable to the traction exerted on the vena cava by the more pendulous condi-

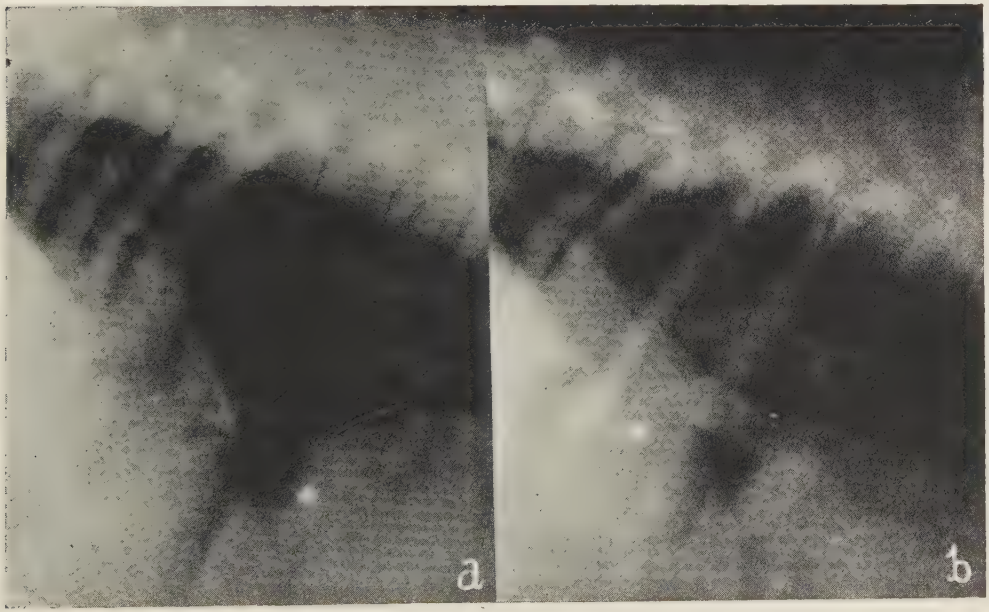

FIG. 2.

Lateral thoracic roentgenograms showing the intrathoracic portion of the inferior vena cava, *a*, during inspiration and *b*, during expiration. Dog standing.

tion of the abdominal viscera when the dog is in the standing position.

Finally we studied the effect of general anesthesia on the diameter of the vena cava. Under anesthesia induced by pentobarbital sodium, the respiration was slow but deep and regular; these were the characteristics desired for this study. Under pentobarbital sodium anesthesia, with the dog lying on its side, no significant change in diameter could be seen during voluntary respiration. Under artificial respiration the diameter during inspiration was about 5% less than that during expiration.

The method of visualizing the vena cava offers distinct possibilities of application to other problems involving the circulatory system. The thoracic portion of the posterior vena cava is anatomically unique since it is the only large vessel lacking a supporting bed of tissues. Consequently, the method employed to visualize it presents two possible sources of error. Adjacent structures, such as the lung, might become adherent to the vein as a result of reaction to operative trauma and the injected substance, causing fixation of the vessel and thus preventing occurrence of changes in diameter. The substance injected might produce sufficient reaction in the wall of the vein to cause it to thicken to such a degree that it would not respond as would a vessel into which nothing had been injected.

In order to evaluate these two possible sources of error, careful necropsy was peformed on some of the animals. The position of the injected region of the vena cava, and adhesions to it, were noted. The vessel was removed and observed both grossly and microscopically. While adhesions from the lung to the vein were noted, the vena cava certainly had not been immobilized in any instance. The wall of the vena cava gave evidence, grossly and microscopically, of a marked reaction having taken place at the site of injection and immediately after it. This reaction subsided so that a few weeks after operation the vein appeared grossly normal. No difference could be detected between a partially filled, injected vena cava and a partially filled vena cava which had not been injected. Slight pressure on the wall of each vessel caused displacement of fluid. Histologically the injected region of the vein could be recognized and some portions of the wall of the vessel remained definitely and probably permanently thickened. The possibility remains that this slight thickening of the wall of the injected vein may have prevented small changes in diameter which might occur in the uninjected vein. This possible source of error does not appear to be of sufficient importance to deter us from making definite conclusions from our observations.

The small changes in diameter of the vena cava of the dog observed by us during respiration do not appear sufficient to produce a significant amount of churning or turbulence regardless of the position of the animal. The churning of the blood described by several workers must be caused by other factors. When the dog is lying on its side changes in diameter are so slight that they may be disregarded. On the basis of this investigation, it appears that thermal and electric contacts with the thermostromuhr unit may be maintained satisfactorily during observations of blood flow, provided care is taken in applying a unit of the proper size. However, turbulence might introduce errors in the values obtained.

Summary and Conclusions. A method of visualizing, roentgenologically, blood vessels such as the vena cava is described. Significant changes in the diameter of the thoracic portion of the posterior vena cava were not observed when the dog was lying on its side. Slight changes in diameter occurred during the respiratory cycle when the dog was standing. The changes in diameter were too insignificant, even in the standing position, to interfere with thermal and electric contacts when the thermostromuhr method of measuring blood flow is used.

10175 P

Mitotic Index of Hyperplastic Interstitial Cells of the Guinea Pig.*

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The interstitial cells of Leydig increase both in number and in size when certain gonadotropic hormones are injected. Mitoses are infrequently seen and the source of the new cells is not known. A study was planned which might give some information on this point.

Seventeen immature guinea pigs (190-230 g) were injected daily with 25 R.U. of PU (Follutein, Squibb) for 1-8 days. Animals were autopsied after 2, 4, 6, and 8 days of injections. On

* Aided by a grant from the Committee for Research in Problems of Sex, National Research Council, administered by Dr. Philip E. Smith.

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the last day of treatment, 0.1 mg of colchicine was injected and the animals sacrificed about 9 hours later. Three guinea pigs were used as colchicine controls and 2 were untreated. The testes and accessories were fixed in Bouin's fluid and stained with haematoxylin and eosin. The average number of Leydig cells per high power field for 130 random samples for each dosage level and mitoses per thousand cells were counted and recorded. In sections of the prostate gland of the same animals, the number of cell divisions per thousand cells was also counted.

In the animals treated with pregnancy urine extract, there is a great increase in the number and size of the cells of Leydig. The degree of response varies with the length of treatment. The data are graphically presented in Fig. 1. Line A of the figure shows the average number of Leydig cells per high power field. In the control testes (Bouin fixation), it is difficult at times to distinguish with certainty the Leydig cells from fibroblasts, histiocytes, and undifferentiated cells in the intertubular mass. This is easily done after hormonal stimulation. The cytoplasm becomes more abundant, acidophilic and granular, while the nucleus becomes more spherical.

An increase in mitotic divisions does not parallel the increase in number of interstitial cells, as can be seen in line C of the graph. These animals have been treated with colchicine, after the Dustin technic. This drug maintains cell divisions at metaphase for about

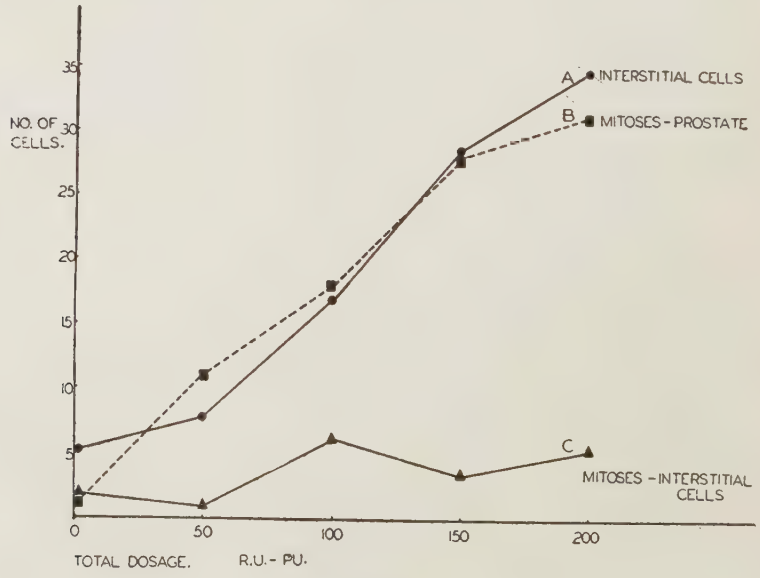

FIG. 1.

9 hours. The number of these mitoses is relatively constant and cannot possibly account for the increase in the number of Leydig cells.

Line B represents the number of mitoses per thousand epithelial cells in the prostate gland of the same animals. Within the duration of the experiment, the longer the treatment with PU, the greater the number of mitoses occurring in a unit of time, showing that these cell divisions will vary directly with the duration of the hormonal stimulation.

Conclusions. The scarcity of cell divisions among the interstitial cells of the testes of PU-treated animals makes unlikely the conclusion that the increase in the number of these cells is due, except to a slight degree, to their mitosis. Observations were presented which support the opinions of Esaki,[1] and others that the interstitial cells have an extrinsic origin. Other data, to be presented elsewhere, indicate that certain intertubular connective tissue elements are differentiated into active secreting Leydig cells by the action of the gonadotropic hormones of pregnancy urine.

10176

Relationship Between Inactivated Prolan and Antiprolan.

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Prolan is a protein-containing hormone in which a protein-like component (carrier) is bound to a comparatively small prosthetic group (active part).[1] While dry prolan powder is very stable prolan solutions are easily inactivated by heat, oxidation and ultraviolet rays[1] although neither ultra-red, red or blue light-rays nor roentgen- or radium-rays affect prolan solutions.[2] The thermo- and photosensitivity of prolan must be due to the presence of the protein-like factor bound to the prosthetic group.

The present investigations concern whether the capacity of prolan to form antiprolan is connected with the prosthetic factor, with

1 Esaki, S., *Z. Mikro. Anat. Forsh.*, 1928, **15**, 368.

1 Von Euler, H., and Zondek, B., *Skand. Arch. Physiol.*, 1934, **68**, 232.

2 Zondek, B., *Hormone des Ovariums und des Hypophysenvorderlappens*, 2nd edition, Springer-Vienna, 1935, p. 252.

its gonadotropic effect, or with the physiologically ineffective carrier substance. Twombly[3] succeeded in inducing antiprolan formation with prolan solutions which had been inactivated to as little as 0.3% of their original effectiveness by one hour's heating in boiling water. This and other facts led the author to the conclusion that antiprolan formation could be explained by the formation of protein antibodies. Brandt and Goldhammer[4] achieved similar results. Gonadotropic hormone which had completely or almost completely lost its hormonal activity through boiling, oxidation or ultraviolet radiation still kept its capacity of inducing the formation of antihormonal sera. The authors conclude that the capacity of forming antihormones is linked to the biologically ineffective protein-like carrier present in the hormone.

I. Our experiments had the reverse result. When we inactivated prolan, traces of the active substance always remained. Even after one hour's boiling at pH 5-7 from 0.3 to 1% activity could still be proved. These minute amounts, however, were sufficient to initiate the formation of antiprolan. Rabbits were immunized for 9 weeks, the animals receiving 250 RU of boiled prolan intravenously twice a week and subcutaneously 4 times a week. The prolan solutions were prepared freshly every day, by dissolving dry powder in water. Before injection the solution was boiled for several seconds. In spite of the boiling there remained 2.5 RU of active substance in the 250 RU of prolan which we used. It had, therefore, been inactivated only 99%. After 9 weeks' injections the rabbits had an antiprolan content of 10 PAU* per cc. Similar experiments carried out with active prolan (250 RU per day) resulted in a much higher titer after 9 weeks, *i. e.*, about 90 PAU per cc. These experiments demonstrate that even the most minute amounts of active prolan are able to form antiprolan, the titer, as a matter of fact, being correspondingly lower (about 11%). We achieved quite different results when we used, instead of a fully effective prolan preparation, a preparation derived from the urine of children which was only slightly effective. Ten mg of this preparation, if injected daily brought about antiprolan formation after 9 weeks. The blood titer was 10 PAU per cc. When, however, we repeated the same experiments with prolan from children's urine which had been boiled for

[3] Twombly, G. H., *Endocrinology*, 1936, **20**, 311.

[4] Brandt and Goldhammer, *Klin. Wchenschr.*, 1938.

* 1 PAU = 1 prolan anti-unit is the smallest amount of the antigonadotropic factor required to annihilate the gonadotropic effect of 1 RU prolan in the immature female rat. At least 10 units should be assayed in a test rat.

3 minutes, even 9 months of injection did not suffice to achieve antiprolan formation. Summarizing, therefore, we can say that immunization with prolan totally inactivated by heat cannot initiate antiprolan formation. If inactivation by heat is incomplete the remaining 0.3-1% of effective prolan is sufficient to bring about antiprolan formation.

II. We used the reverse approach in our further experiments. If prolan whose prosthetic group has been destroyed by boiling is said to be able to form antiprolan, prolan which has been inactivated by boiling must be able to bind itself to antiprolan, *i. e.*, to neutralize antiprolan. This, however, is not true, as shown by the following experiments: 100 RU of prolan, heated for one hour at pH 6 was kept in the incubator for 2 hours with 15 PAU of antiprolan.† The 100 RU of boiled prolan could not inactivate the 15 PAU. This was demonstrated by the fact that 10 RU of active prolan which had been added to the mixture (100 RU of boiled prolan + 15 PAU of antiprolan) was inactivated after having been kept in the incubator for 2 hours.

A quantitative experiment is illustrated in Table I.

TABLE I.

Infantile rat	Anti-prolan	Boiled prolan	Active prolan	Gonadotropic reaction	Binding effect of boiled prolan
		A. Control Experiments.			
	mg	RU	RU		
R.3261	—	100	—	—	
R.3262	15	—	12	—	
R.3263	15	—	14	—	
R.3264	15	—	16	—	
R.3265	15	—	18	—	
R.3266	15	—	20	+	
R.3267	15	—	22	+	
		B. Main Experiments.			
R.3255	15	100	12	—	<8 RU
R.3256	15	100	14	—	<6 RU
R.3257	15	100	16	—	<4 RU
R.3258	15	100	18	—	<2 RU
R.3259	15	100	20	+	
R.3260	15	100	22	+	

In the control experiment (R. 3262-3267) the titer of 15 mg antiprolan dry powder was determined. It became apparent that 15 mg of antiprolan was able to inactivate 12-18 RU of prolan (R. 3262-3265) while 20 RU was not inactivated, for R. 3266 showed oestrous reaction (HVR I). Fifteen mg of the antiprolan

† For our experiments we used our antiprolan-acetone-dry powder (Zondek and Sulman, PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 708), which was freshly dissolved in water for every experiment. 1 mg = ca 1 PAU.

which we used contained, therefore, 18 to 19 PAU. A further control experiment revealed that 100 RU of prolan, boiled for one hour at pH 6, no longer brought about the gonadotropic reaction in the infantile rat and had, therefore, been inactivated. (R. 3261.) In the main experiment each time a solution of 15 mg of antiprolan in 1 cc of distilled water, was combined with 100 RU of boiled prolan, dissolved in 2 cc of distilled water, and placed in the incubator for 2 hours. Then increasing quantities of active prolan (12-22 RU), dissolved in 1 cc of water, were added and kept in the incubator for another 2 hours. The total solution (4 cc) now contained 18-19 units of antiprolan, 100 units of prolan inactivated by boiling and 12-22 units respectively of active prolan. The total solution was injected into infantile rats (6 portions in the course of 36 hours) and the gonadotropic reaction was recorded. The experiments showed that in rats Nos. 3255-3258 the vagina had remained closed, that there was no oestrogenic reaction whatsoever and uteri as well as ovaries had remained absolutely infantile. Thus we saw that 18-19 antiprolan units were able to inactivate 12-18 RU of active prolan in spite of the fact that 100 RU of inactivated prolan had been added. Prolan which had been inactivated by boiling, therefore, did not unite with antiprolan in any way whatsoever. One hundred units of boiled prolan could not bind a few units of antiprolan, for this would have been sufficient to produce the prolan reaction.

After 96 hours R. 3259 and R. 3260 showed the gonadotropic reactions (open vagina, oestrous vaginal smear, thick uteri and enlarged ovaries with corpora lutea). In these experiments prolan had been given the opportunity of being effective because there was an excess of it: 20 or 22 RU of prolan were not completely inactivated by 18-19 PAU of antiprolan; some units of active prolan remained and it was those which brought about the gonadotropic reaction.

The above experiments, therefore, demonstrate that prolan inactivated by boiling completely loses its capacity of neutralizing antiprolan.

Conclusion. Boiled prolan is still able to initiate antiprolan formation for one hour's boiling leaves 0.3-1% active substance. These minute amounts of prolan are sufficient for the formation of antiprolan. After complete inactivation through heat prolan is neither able to produce antiprolan *in vivo* nor paralyze antiprolan *in vitro*. Since prolan consists of an active prosthetic group and an inactive carrier (Euler and Zondek '34) we conclude: The capacity of prolan

to initiate the formation of antiprolan through protracted treatment and the capacity of paralyzing antiprolan *in vitro* is immanent in the active prosthetic group and not in the carrier substance which is hormonally ineffective.

10177

Metabolic Properties of the Regions of the Amphibian Gastrula.

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A number of authors have investigated the oxygen consumption and carbon dioxide production of the amphibian embryo at various stages of its development, with results of considerable interest. But such investigations throw no light upon the properties of the individual regions of the embryo at various stages, although the work of experimental embryologists has furnished us during the last 20 years with fundamental information about these regions and the part they play in the morphogenesis of the organism. In particular, the phase of gastrulation, during which are formed the germ-layers of classical embryology, and which involves the action of the primary organiser in determining the main axial structures of the embryo, merits the closest study.

Only recently have sufficiently delicate chemical methods become available for attacking this problem. Since Rehberg[1] developed the first ultra-micro burette, these methods have been greatly extended by the work of Linderstrøm-Lang, Holter, and their collaborators at Copenhagen. The first application of this kind of technic to the metabolism of the gastrula was made by Heatley,[2] who estimated the amounts of glycogen in the various regions of the gastrula and confirmed by direct chemical analysis the specially marked disappearance of this polysaccharide during the invagination of the roof of the archenteron, through the dorsal lip of the blastopore. All such observations have significance since it is in the dorsal lip of the blastopore and nowhere else during normal development, that the organiser "hormone" is liberated from its inactive combined form.

Wishing to study the metabolic properties of the dorsal lip of the blastopore, as opposed to the ventral ectoderm, where the organiser

1 Rehberg, P. B., *Biochem. J.*, 1925, **19**, 270.
2 Heatley, N. G., *Biochem. J.*, 1935, **29**, 2568.

is not normally liberated, by manometric means, we found in the Cartesian Diver ultra-micro-manometer a very suitable tool. The suggestion that the Cartesian Diver could be used for such a purpose we owe to Linderstrøm-Lang,[3] but he himself has not so far developed it for use with living tissues. If a bubble of air is enclosed in a small open glass bulb so that it floats within a larger vessel, the buoyancy of the diver will vary according to the pressure imposed on the whole system, and it will sink or rise as this pressure rises or falls. Conversely, if the gas phase in such a diver is increased or diminished by the process of a reaction within it, the pressure required to maintain it at a given level will correspondingly rise or fall. In this way the diver is equivalent to a constant-volume manometer, and we have found that a suitably modified formula of the Warburg type may be used for calibration, the volume of the diver being known. The divers used are readily made from Pyrex capillary tubing and consist of a neck, a bulb, and a tail to ensure that the diver floats upright. The neck varies from 6-16 mm in length according to the experiment which it is desired to make, and the total diver volume from 20-40 mm^3. It is desirable, in order that the loss of gas from the diver should be minimal, to use a strong salt solution as the flotation medium and since we wished to measure the ammonia produced during the experiment as well as the total nitrogen of the piece of tissue used, we substituted lithium chloride at the same density, for the saturated ammonium sulphate of Linderstrøm-Lang. Ammonia was estimated by the method of Linderstrøm-Lang and Holter[4] and total nitrogen by a new ultra-micro-Kjeldahl method devised by us (Needham and Boell[5]). The tissues used were for the most part single dorsal lips of gastrulæ and single pieces of ventral ectoderm, dissected out, often from the same embryo, by the Spemann glass needle technic Such pieces weigh less than 100 γ dry weight.

The delicacy of the method in its present form may be gauged by the fact that whereas 1 cm on the scale of the usual Warburg manometer corresponds to a gas change of about 20 mm^3, 1 cm on the scale of the diver manometer corresponds to a gas change of from 0.007-0.015 mm^3.

For the measurement of anaerobic glycolysis, the tissue lies in a film of Holtfreter-bicarbonate solution at the bottom of the bulb,

[3] Linderstrøm-Lang, K., *Nature*, 1937, **140**, 108.

[4] Linderstrøm-Lang, K., and Holter, H., *Comptes Rend. Trav. Lab. Carlsberg*, 1933, **19**, 1.

[5] Needham, J., and Boell, E. J., *Biochem. J.*, in press.

the gas space is filled with 95%N_2/5%CO_2 gas mixture, and the diver is sealed with the oil seal. Completely anaerobic conditions can only be attained by handling the divers within a special glass chamber with pipettes mounted on universal glass joints. A stream of the purified gas mixture passes through the chamber, and the divers are transferred from chamber to vessel carrying drops of lithium chloride solution which serve to protect them from the air during transit. The lithium chloride and the oil are saturated with the gas mixture beforehand. With these methods, an extensive set of measurements on the embryos of *Rana temporaria* showed a substantial difference between the organiser region and the ventral ectoderm.

	N_2/Q_L	NH_3 (cu mm $\times$ $10^{-3}/\gamma$ dw/5 hr)
Dorsal lip of blastopore	0.63	2.31
Ventral ectoderm	0.21	0.97

Another series of experiments on *Triton alpestris* gave a similar, though not quite so large, difference.

For the measurement of oxygen consumption, Holtfreter solution without bicarbonate was used and the neck of the diver was coated with paraffin wax. It was thus easy to suspend a drop of alkali at the lower end of the neck, just above the bulb, for the absorption of the CO_2 produced. Two series of experiments were performed, one on the embryos of *Discoglossus pictus,* the other on those of *Amblystoma mexicanum.* In neither case could any appreciable difference between the oxygen consumption of the dorsal lip and that of the ventral ectoderm be observed.

	Q'_{O_2} (related to total N, not dry wt)	
	Discoglossus	*Amblystoma*
Blastula roof	—	2.6
Dorsal lip of blastopore	4.8	3.2
Ventral ectoderm	4.8	3.2
Closing neural folds	3.0	—

For the measurement of respiratory quotient, a diver of larger volume and with a longer neck is used. The neck is paraffin waxed, as for oxygen consumption, but contains a series of drops or films in the following order: nearest the bulb, the drop of alkali, then a drop of acid sufficient to give a great excess of acid if mixed with the other solutions and tissue, then a further drop of acid in direct contiguity with the oil drop. At first the diver becomes steadily heavier owing to the absorption of oxygen by the tissue and of carbon dioxide by the alkali, but after a suitable time, usually here

3 hr, the system is subjected to a pressure of about a foot of mercury, which has the effect of blowing down the drops of alkali and acid into the bulb, the oil drop then returning to its original position, and the diver quickly becoming much lighter owing to the liberation of carbon dioxide. For the bound CO_2 of tissue and solutions a second diver is mixed at the beeginning of the experiment. The method is thus entirely analogous to that of Dickens and Simer,[6] who used annular cups on Warburg manometers.

As a result of experiments with this method, it seems that during gastrulation the dorsal lip of the blastopore shows a much greater trend towards unity than the ventral ectoderm. As was expected from the work of Brachet,[7] blastula roof gave a low quotient (0.75) and closing neural folds a high one (unity). Intermediate average values were as follows:

	Amblystoma mexicanum R.Q.
Blastula roof	0.75
¼-moon yolk-plug gastrula:	
Dorsal lip of blastopore	1.0
Ventral ectoderm	0.83
½-moon yolk-plug:	
Dorsal lip of blastopore	1.0
Ventral ectoderm	0.89
Closing neural folds	1.0

We are not yet in a position to say definitely whether the ventral ectoderm ever reaches a respiratory quotient of unity before it is completely underlain by invaginating mesoderm.

We should like to postpone the discussion of the meaning of these differences until the full publication of the data. In the meantime, we would draw the attention of biologists in general as well as embryologists to the existence of the very delicate and reliable ultra-micro-manometer now available in the Cartesian diver.

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[6] Dickens, F., and Simer, F., *Biochem. J.*, 1930, **24**, 905.

[7] Brachet, J., *Arch. Biol.*, 1934, **45**, 611.

10178

Maintenance of Fibroblasts in Artificial and Serumless Media.

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The importance of developing artificial media, which can be used in place of serum for maintaining the life of organs and tissues outside the body, hardly needs to be emphasized. Many of the studies for which the organ-culture technic[1] was invented, as well as others that can be carried out by the simpler methods of tissue-culture, depend for their success on the creation of suitable artificial media. These media are needed to reduce the cost of experimentation, to make possible extensive work with human organs and those of small animals from which serum in large quantity cannot be obtained, and for all studies in which the production of serum and other protein substances is to be investigated. For cultivating organs and for all work with tissues in which function rather than growth is the subject of study, it is important that these media maintain the cells without causing proliferation. All the artificial media previously reported have been designed to promote growth.[2,3] The media to be described in this paper were designed for maintenance. One of them is serumless. In the others, a very small amount of serum has been incorporated. The results obtained when these media were used to maintain a pure strain of fibroblasts in tissue-culture are described below. Experiments in which they were used for cultivating organs will be reported in another communication.[4]

The compositions of the media are as follows:

MEDIUM I.

Whole-blood digest to give either 30 or 60 mg % nitrogen
Serum, 2 or 3 %
Tyrode's solution

MEDIUM II.

Whole-blood digest to give either 30 or 60 mg % nitrogen	per 100 cc	
Cysteine hydrochloride	9.0	mg
Insulin	0.1	unit

1 Carrel, A., and Lindbergh, C. A., *Science*, 1935, **81**, 621; Lindbergh, C. A., *J. Exp. Med.*, 1935, **62**, 409; Carrel, A., *J. Exp. Med.*, 1937, **65**, 515.

2 Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, **44**, 503; 1928, **47**, 353, 371; 1928, **48**, 533; Baker, L. E., *J. Exp. Med.*, 1929, **49**, 163; *Science*, 1936, **83**, 605.

3 Vogelaar, J. P. M., and Erlichman, E., *Am. J. Cancer*, 1933, **18**, 28.

4 Some of this work on media for organ-cultivation has already been incorporated by Carrel, A., and Lindbergh, C. A., in *The Culture of Organs*, New York, Paul B. Hoeber, Inc., 1938.

Thyroxine	0.001 mg
Hemin	0.004 "
Vitamin A (containing some D)	100.0 units
Vitamin B_1	0.1 gamma
Vitamin B_2	3.4 "
Ascorbic acid	0.3 mg
Glutathione	1.2 "
Glucose	200.0 "
Potassium iodide	0.13 "
Salts as in Tyrode's solution	

To bring the vitamin A into solution, it was necessary to dissolve it at high concentration in serum and then use a small amount of this serum in the medium.[5] The concentration required proved to be only 0.07%.

Many of the substances in this medium were selected because they had previously been found by Baker,[2] or by Vogelaar and Erlichman,[3] to prolong the life of cells in artificial growth-promoting media. The concentrations of the individual constituents have been adjusted to those that seemed best suited to maintenance.

MEDIUM III.

This contained all the constituents listed under Medium II and in addition the following in 100 cc:

Tryptophane	5 to 10.0 mg
Witte's peptone to give	6.0 " N
Sodium glycerophosphate	57.5 "
Urea	2.4 "
Glycerine	0.2 cc
Thymus nucleic acid*	20.0 mg
Antuitrin	0.2 cc
Adrenalin chloride (1 to 1000)	0.1 "
Eschatin (suprarenal cortex hormone)	0.1 "
Pitressin (pituitary hormone)	0.1 "

This medium also contained some serum, about 0.07%, needed to dissolve the vitamin A.

MEDIUM IV.

This medium contained no vitamin A and no serum. Otherwise, its composition is the same as Medium III.

The blood digest was prepared from whole bovine blood, which was first incubated with chloroform to destroy the antienzyme, and then digested with pancreatin. All undigested proteins and the growth-promoting proteoses formed from them were removed with trichloracetic acid. Then the filtrate was boiled to destroy the remaining trichloracetic acid and drive off the chloroform. The resultant mixture was made isotonic and adjusted to pH 7.4. It was found on analysis to have a ratio of amino to total nitrogen of 0.45.

The procedure used in testing these media for their ability to maintain fibroblasts was as follows: Cultures from a pure strain of

[5] Baker, L. E., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 124.

* Dr. P. A. Levene of The Rockefeller Institute kindly prepared and furnished this substance.

chicken-heart fibroblasts were embedded in Carrel flasks (D-3) in 1 cc of coagulum containing 25% plasma. To get rid of the serum in this coagulum, the cultures were washed on the following day and every 2 days thereafter for 2 hours at 37°C with a large volume of the medium. Then this wash fluid was withdrawn and 0.5 cc fresh medium was supplied. Under this treatment, the serum originally present in the coagulum disappeared in about 2 weeks. The washing was continued, nevertheless, throughout the entire period of cultivation. To ascertain the effect of the various media, the cells were examined microscopically at frequent intervals. At the end of the cultivation-period, which extended from 42 to 56 days, the vitality of the cells and their ability to proliferate were tested by transplanting them into a growth-promoting medium. A sister colony was cultivated in each case in some control medium, the nature of which is indicated below.

Medium I, when tested in this manner, was found to be an excellent maintenance-medium. Fibroblasts cultivated in it were maintained in excellent condition for 42 days. During the first few days, *i. e.,* before all the serum had been washed out of the coagulum, the cells proliferated at a very slow rate. After the serum of the coagulum was removed, they were maintained with little or no proliferation. Then, when growth-promoting substances were added at the end of cultivation, they proliferated again. Control cultures kept in blood digest and Tyrode's solution died soon after all the serum had been removed from the coagulum.

Chicken-heart fibroblasts cultivated in Medium II lived for 50 days. They remained in better condition and outlived sister cultures kept in blood digest alone. But toward the end of the experiment, the cells in the experimental medium became scattered and began to look starved. As it seemed probable that longer cultivation in this medium would not be feasible, the colonies were transferred to a new coagulum and given growth-promoting nutrients. Active proliferation ensued. It would seem, therefore, that this medium can maintain the cells for a considerable time, but not indefinitely.

Medium III was devised in an attempt to improve Medium II. Four of the 10 new constituents added, antuitrin, peptone, tryptophane, and sodium glycerophosphate, when tested separately were found to improve the nutritive and maintenance values of Medium II. The other 6 gave indications of being beneficial, but the magnitude of the results obtained, when each was tested separately, was too small to constitute definite proof that they were essential. A comparison of Medium II and Medium III made on sister cultures of fibroblasts

showed that the cells cultivated in Medium III invariably remained in better condition and also outlived those cultivated in Medium II. When transferred after 56 days of cultivation to a growth-promoting medium, all the colonies cultivated in Medium III proliferated again, while only 25% of those cultivated in the simpler medium were found to be capable of renewed growth. In one experiment, some colonies that had been cultivated for a month by the procedure outlined above were left for another month without change of fluid. At the end of this time, the cells were still found to be in good condition and able to proliferate.

But Medium III is not serumless. In order to incorporate some vitamin A in it, 0.07% of serum had to be used. Vitamin A was added to this medium because it is always present in serum and has also been found a necessary constituent of artificial growth-promoting media. But no evidence that it was essential for maintenance has been obtained. Hence, to ascertain if this vitamin and the serum used to dissolve it could be eliminated, sister colonies of fibroblasts were cultivated in Medium III, with and without vitamin A. All the differences observed were in favor of the serumless medium. The cells in this medium seemed to be a little clearer and in better condition throughout the entire period of their cultivation. After 62 days, the colonies that had been carried in the serumless medium were transferred to a new coagulum and given growth-promoting nutrients. They responded by growing actively.

To summarize: Four media have been described in which fibroblasts in pure strain have been maintained in vital condition and with little or no proliferation for periods varying from 42 to 56 days. The first of these media is simple, inexpensive, and easy to prepare. The last is serumless.

10179 P

Simultaneous Distemper and Lymphocytic Choriomeningitis in Dog Spleen and the Sparing Effect on Poliomyelitis.

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While continuing the study of canine distemper in monkeys, evidence accumulated which indicated that our virus-source material

was a mixture. This has been verified by the recovery of the virus of lymphocytic choriomeningitis from 4 different samples of dog spleen containing the virus of canine distemper and by its presumptive demonstration in 7 other instances. The present report submits evidence that the virus of lymphocytic choriomeningitis occurs in the dog and to correct our earlier description of canine distemper in the monkey and the sparing effect of distemper on experimental poliomyelitis.[1, 2]

Three bacteriologically sterile 20% suspensions of pooled dog spleens taken from separate harvests of canine-distemper virus of the same strain were inoculated intracerebrally in 0.03 cc amounts into groups of mice. A uniform clinical response resembling Traub's description of lymphocytic choriomeningitis followed.[3] Part of one suspension was inoculated subcutaneously into 2 puppies. Fifteen days later these were moribund with canine distemper and were sacrificed. Six mice inoculated with splenic emulsion of one of these puppies also sickened and died. Fourteen of 18 examined mice had lymphocytic chorioiditis.

At the same time a considerable number of mice from the same colony were inoculated intracerebrally with dog-spleen suspensions

TABLE I.
Tests for the Recovery of Virus of Lymphocytic Choriomeningitis.

Mice injected with distemper dog spleens from various serial passages of the original strain.		
No. of Mice	Material Pool No.	Day of death of mice
12	1	5, 5—, 7—, 7+, 8, 8+, 9, 9+, 10+, 12, *12, 12*
10	1 (intraperitoneal)	6, 8, 8, 9, 10, 11, 14, S, S, S
6	1*	8, 8+, 8+, 8+, 11+, 11
8	2	3, 8+, 8+, 9—, 9+, *9, 9, 9*
8	3	1, 8, 8+, 8+, 10—, S, S, 10+
Mice injected with distemper dog spleens from other sources and with other materials.		
No. of Mice	Material Pool Strain	Day of death of mice
6	"B"	S, S, S, S, S, S
8	"C"	S, S, S, S, S, S, S, S
6	"D"	S, S, S, S, S, S
14	Sterile broth	S, S, S, S, S, S, S, S, S, S, S, S, S, S

All injections given intracerebrally. Pool No. 1 also given intraperitoneally. * indicates the spleen of a puppy which had been inoculated with Pool No. 1 15 days previous and which was followed by severe distemper. The + and — signs indicate that lymphocytic chorioiditis was or was not present. The *italics* indicate mice sacrificed when moribund for supply of virus.

1 Dalldorf, G., Douglass, M., Robinson, H. E., *J. Exp. Med.*, 1938, **67**, 333.

2 Dalldorf, G., Douglass, M., Robinson, H. E., *J. Exp. Med.*, 1938, **67**, 323.

3 Traub, E., *J. Exp. Med.*, 1936, **63**, 533.

secured from other sources and containing canine-distemper virus, with various other suspected materials and with sterile broth. All remained well. The data of representative groups have been incorporated in Table I as evidence both of the freedom of our stock animals from spontaneous lymphocytic choriomeningitis as well as the presence of a virus, capable of producing the clinical response of lymphocytic choriomeningitis, in the original materials.

Three guinea pigs were inoculated subcutaneously (0.5 cc) with one of the pooled dog-spleen suspensions and developed the symptoms of lymphocytic choriomeningitis, emaciation, dyspnea, and conjunctivitis. All 3 died. Two of these were demonstrated to have the lesions of lymphocytic chorioiditis (heavy round-cell infiltrate of the chorioid plexus and liver).

A fifth sample of pooled dog-spleen from the first series produced a severe disease in monkeys having the features described by Armstrong[4] as occurring in lymphocytic choriomeningitis. Splenic samples of 3 of these monkeys were injected into mice and produced the symptoms and lesions of lymphocytic choriomeningitis. Virus was also demonstrated in the blood of 2 of the monkeys. One blood sample was injected intravenously into 6 mice of which 4 survived. These were reinoculated intracerebrally 41 days later with known lymphocytic choriomeningitis virus received from Dr. T. M. Rivers and were found resistant while controls all died.

A sixth sample of pooled dog-spleen was injected subcutaneously into a young rhesus monkey which sickened but recovered; 105 days later this animal was inoculated intracerebrally (0.5 cc of a 10% suspension) with known lymphocytic-choriomeningitis virus and showed no response.

A number of other observations, in particular certain adrenal inclusion-bodies, lead us to believe that all of the material we have been using as a source of canine-distemper virus has been contaminated. This has led to a trial of the sparing effect of lymphocytic-choriomeningitis virus in rhesus monkeys intracerebrally inoculated with poliomyelitis virus. In these experiments the same methods and materials have been used as in our original reports with the exception that suspensions of guinea-pig brain taken from animals infected with a known strain of lymphocytic-choriomeningitis virus or the strain recovered from dog spleen and repeatedly passaged through monkeys were used instead of splenic suspensions from dogs or ferrets with distemper. The results have been summarized in Table II.

Since the virus of lymphocytic choriomeningitis has been repeat-

[4] Armstrong, C., *Pub. Health Rep., U. S. P. H. S.*, 1934, **49**, 1019.

TABLE II.

Sparing Effect of Lymphocytic Choriomeningitis on Experimental Poliomyelitis.

No. of Animals		Symptoms predominantly those of		Lesions predominantly those of		Outcome	
		Polio.	Chorio.	Polio.	Chorio.	Recovered	Died
8	Lymph. chor. virus injected before or simultaneously with inoculation with poliomyelitis	0	8	0	3	4	4
10	Lymph. chor. virus injected during incubationary period of poliomyelitis	5	5	3	4	3	7
12	Lymph. chor. virus injected after the appearance of preparalytic stage	11	1*	6	3	5	7
23	Poliomyelitis, controls	20†		7	0	5	18
6	Lymph. chor. controls	0	6			1	5

*Shortly after the second virus was injected the fever, irritability, and other signs of poliomyelitis disappeared.

†Three controls were cases of "missed infection."

edly found in the passage distemper-material we have used and was presumably present in our original experiments and since it is capable of producing a sparing effect on experimental poliomyelitis it seems advisable to report these results. Unfortunately we are not yet able to state definitely what part, if any, canine distemper played in the original work. This as well as extended study of the significance of lymphocytic choriomeningitis in the dog, its distribution and possible significance in the dissemination of the disease are all to be studied. Further investigation of the sparing effect of lymphocytic choriomeningitis on experimental poliomyelitis is also in progress.

10180 P

Effect of Prolonged Theelin Injections on Transplantable Mammary Adenofibroma.*

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We previously reported that theelin (estrone) administered to male white rats did not affect the growth rate of a transplantable mammary adenofibroma, that it failed to arrest fibrosis of these tumors and significantly retarded the normal body weight gains.[1] For a comparative study, 50 i.u. of aqueous theelin† was administered subcutaneously bi-weekly over a period of 165 to 368 days to 2 groups of male white rats implanted with mammary adenofibroma, Strain 1-a, morphologically and functionally similar to the tumor strain previously tested.[2]

Failure of transplant "take" occurred in 4 of 7 controls and 7 of 12 theelin-treated animals in the 300-day group; while in the 160-day-old animals, 3 of 8 controls and 6 of 12 theelin-treated failed to

TABLE I.

Mean Daily Changes in Body Weight (g/day).

Group I, 300-day Controls vs. 160-day Controls; Group II, 300-day Theelin vs. 160-day Theelin; Group III, 300-day Controls vs. 300-day Theelin; Group IV, 160-day Controls vs. 160-day Theelin.

Group	300 d. Control	160 d. Control	300 d. Theelin	160 d. Theelin	Diff.	P*
I	+.149	+.304			+.155	.3
II			—.002	+.227	+.229	.042†
III	+.149		—.002		—.151	.4
IV		+.304		+.227	—.077	.3

* P = probability of a difference as great or greater than that obtained occurring by chance alone. See Fischer, R. A., "Statistical Methods for Research Workers," London, Oliver & Boyd, 1934, 5th Ed., Chap. V.

† Taken by itself, this might be of significance, although on the borderline. But in view of the other high values for P, it must be regarded with suspicion.

* Aided by grants from the Rockefeller Fluid Research Fund of Stanford University School of Medicine, and from the Scientific Research Committee of the American Medical Association.

† We are grateful to Parke, Davis and Company for the donation of theelin used in this experiment.

1 Emge, L. A., Murphy, K. M., and Schilling, Walter, PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 21.

2 Emge, L. A., *Arch. Path.*, 1938, **26**, 429.

TABLE II.

Mean Daily Changes in Tumor Weight (g/day).

Group I, 300-day Controls vs. 160-day Controls; Group II, 300-day Theelin vs. 160-day Theelin; Group III, combined Controls vs. combined Theelin.

Group	300 d. Control	160 d. Control	300 d. Theelin	160 d. Theelin	Diff.	P
I	+.016	+.055			+.039	.5
II			+.053	+.023	—.030	.3
III‡	+.041		+.037		—.004	.9

‡ It is proper to combine these into one group, since there is no significant difference between the control animals and between the theelin-treated animals.

grow tumors. Mean daily body and tumor weight changes are recorded in Tables I and II.

It is evident that the effect of injections of 50 i.u. of aqueous theelin bi-weekly over a period of 165 to 368 days is not of statistical significance in relation to the growth of adenofibroma, Strain 1-a, and the body weight.

A microscopic study revealed that the tendency to change from adenofibroma to fibroma was not arrested by theelin. Wherever glandular tissue was preserved, ducts were found to predominate. A hyperplasia of ducts similar to Schimmelbusch's disease was seen occasionally. Unusual hyperplasia was noted in but one tumor of the 160-day theelin-treated group.

From the tumors removed in from 165 to 368 days, autotransplants were made into the opposite groin and theelin treatment continued as before for 74 days. Tumors derived from these implants grew slowly, with a further loss of adenomatous components and a marked tendency toward hyalinization.

Conclusions. Bi-weekly injections of 50 i.u. of aqueous theelin over a period of 165 to 368 days in male white rats implanted with adenofibroma did not prevent these tumors from undergoing fibromatous changes except in one tumor growing in a 160-day-old theelin-treated animal, which underwent massive glandular hyperplasia. A reduction in body weight after large doses of theelin, as reported previously, did not occur in either age group with smaller amounts given over longer periods, nor did the amount of theelin affect the daily growth rate of adenofibroma, Strain 1-a.

10181

Succinic Acid and Glucose in Pituitary Ketonuria.

A. W. Terrell. (Introduced by F. D. W. Lukens.)

From the George S. Cox Medical Research Institute, University of Pennsylvania, Philadelphia.

Deuel, Murray and Hallman[1] have shown that sodium succinate is ineffective in preventing the ketonuria in fasting rats previously fed a high fat diet. Since Rietti[2] has shown that the ketonuria of pancreatic diabetes is reduced by hypophysectomy and since extracts of the anterior pituitary produce ketonuria in the fasted rat, a study of the effect of succinic acid in pituitary ketonuria was undertaken.

Male albino rats weighing between 180 and 200 g were fasted for 3 days in metabolism cages and the urine collected in 20% copper sulphate. Each 24-hour volume was analyzed for total acetone bodies by the method of Van Slyke. The results are expressed in mg per 100 g of body weight of rat per day.

On the 2nd and 3rd day of fasting, anterior pituitary extract was given subcutaneously, 0.5 cc per 100 g, and 2 cc of physiological saline was given intraperitoneally to insure a satisfactory urine volume. Since about 25% of our rats failed to respond well to pituitary extract, only those rats which developed satisfactory ketonuria were used. Among these animals there was a variation in the degree of acetonuria, but since the same group of animals, 17 in number, was used repeatedly for the 3 experiments, this factor is controlled. Between each period of food withdrawal and extract treatment the animals were allowed to regain their initial weight. After the first experiment with fasting and extract, the effect of sodium succinate, food withdrawal and extract was examined. The succinate was given orally for 4 days before as well as during the fasting period. The dose of sodium succinate was 20 mg per 100 g of rat per day which corresponds on a body-weight basis with the dose used clinically by Koranyi and Szent-Györgyi.[3]

In the third series of experiments the same dose of glucose was used (20 mg per 100 g per day).

The results are summarized in Table I, and show the average acetone body excretion for 100 g of rat per day for the 2nd and 3rd

1 Deuel, M. J., Jr., Murray, S., and Hallman, L., Proc. Soc. Exp. Biol. and Med., 1937, **37**, 413.

2 Rietti, C. T., *J. Physiol.*, 1932, **77**, 92.

3 Koranyi, A., and Szent-Györgyi, A., *Deutsch. med. Wchnschr.*, 1937, **63**, 1029.

days of extract injection. The reduction of acetonuria associated with succinate and glucose administration is indicated. This diminution is statistically significant except for the 3rd day of succinate. The fact that glucose is more efficient and the fact that succinic acid may be converted to glucose in the body[3] suggest that sodium succinate may exert its effect because of its conversion to glucose.

TABLE I.

Series	No. of Rats	Avg wt	Wt loss, avg	Avg acetone body excretion in mg per 100 g body wt† day of fast		
				1	2*	3*
A	17	185.1	23.8	1.24	21.28 ± 1.80	23.41 ± 2.21
B	17	183.6	26.4	0.94	15.89 ± 0.93	20.95 ± 0.84
C	17	189.2	21.3	1.01	8.74 ± 0.47	10.46 ± 0.52

A = Fasting.
B = Fasting with Succinate
C = Fasting with Glucose.
* Given anterior pituitary extract 0.5 cc per 100 g body weight.
† With standard error of mean.

These results are comparable to the effects of succinic acid and glucose on the ketonuria produced by high fat diets.[1] It appears that experimental ketonuria produced in 2 ways, by high fat diets or by fasting and anterior pituitary extract, may be diminished by succinic acid, but that this substance acts much less effectively than glucose.

10182

Availability of Dibenzoylcystine for Growth of the Young White Rat.

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If the α-amino group of cystine was blocked by replacement of a hydrogen atom with a benzoyl group, the sulfur of the resulting dibenzoylcystine was not oxidized readily to sulfate sulfur in the organism of the rabbit, but a large part of the compound was excreted by the kidneys, either unchanged or as the corresponding cysteine derivative.[1] Since the biological oxidation of this α-substi-

3 Ringer, A. I., Frankel, E. M., and Jonas, L., *J. Biol. Chem.*, 1913, **14**, 539.

1 Lewis, H. B., Updegraff, H., and McGinty, D. A., *J. Biol. Chem.*, 1924, **59**, 59.

tuted cystine derivative to sulfate was almost negligible after its parenteral administration, while the oxidation of the sulfur of dibenzoylcystine after oral administration, although variable and not marked, was considered significant, it was suggested that orally administered dibenzoylcystine was hydrolyzed in part to yield cystine and benzoic acid, prior to absorption from the alimentary tract, by microbial or other agencies.

A second method of attack on this problem is the study of the supplementary effect of dibenzoylcystine in the diet on the growth of young white rats fed a diet known to be deficient in its content of sulfur-containing amino acids. The utilization for purposes of growth of a cystine derivative is usually assumed to be evidence of the conversion of the derivative to cystine in the body, although this interpretation is not necessarily the only possible one. Jones, Andrews and Andrews[2] reported that rats fed dibenzoylcystine as a supplement to a basal diet low in its cystine content showed occasional irregular increases in the rate of growth in comparison with control animals receiving the basal diet. This suggested that hydrolysis of dibenzoylcystine might proceed under certain conditions with sufficient rapidity to permit this cystine derivative to serve as a source of cystine for purposes of growth of the young white rat.

Since any significant biological hydrolysis of dibenzoylcystine would result in the liberation of benzoic acid, which may influence unfavorably the growth of rats, especially when diets low in protein (casein) are fed,[3] it seemed that optimal conditions for the observation of any growth-promoting effect of dibenzoylcystine might be obtained, if glycine, a non-essential amino acid, were also added to aid in the detoxication of any benzoic acid produced by the hydrolysis of the dibenzoylcystine. The presence or absence of glycine in the diet should not influence the rate of growth, if benzoic acid were not a dietary component[3] as such or formed in the metabolic processes.

Young white rats in litter units were fed a basal diet low in its content of cystine[4] for a preliminary feeding period of 14 days. The animals either failed to gain or showed almost negligible increases in weight. Water-soluble and fat-soluble vitamins were

2 Jones, J. H., Andrews, K. C., and Andrews, J. C., *J. Biol. Chem.*, 1935, **109**, xlviii.

3 Griffith, W. H., *J. Biol. Chem.*, 1929, **82**, 415.

4 White, A., *J. Biol. Chem.*, 1936, **112**, 503. The diet was altered to contain 5% instead of 6% of casein (Labco Brand, vitamin-free) and 1% of agar.

supplied by the separate administration of dried brewer's yeast (approximately 400 mg) and cod liver oil (3 drops) daily. After the preliminary feeding period, the animals were divided into 3 groups and the paired feeding method employed. One group received supplementary cystine (0.5%); a second group received dibenzoyl-*l*-cystine[5] in an amount equivalent in sulfur content to 0.5% of cystine; and the third group was continued on the basal diet. Glycine (0.5%) was added to the diets of all 3 groups. The experimental period included 50 days.

TABLE I.

Food Consumption and Increases of Weight of Rats Fed a Basal (Cystine Deficient) Diet Supplemented by Cystine and Dibenzoylcystine Over a Period of 50 Days.

Glycine was supplied in all the diets. All values are expressed as grams. The letters after the numbers of the animals indicate sex. The pairings for the controlled feedings were as follows: Rats 4211, 4413 and 4119; 4225, 4424 and 4127; 4238 and 4436.

Supplement	Cystine			Dibenzoylcystine			None	
Rat No.	4211M	4225F	4238F	4413M	4424F	4436F	4119M	4127F
Initial wt	51	53	54	59	59	61	75	50
Final wt	117	116	117	79	75	66	96	66
Total gain	66	63	63	20	16	5	21	16
Avg daily gain	1.32	1.26	1.26	0.40	0.32	0.10	0.42	0.32
Total food	275	278	289	282	286	297	278	283
Avg daily food	5.5	5.6	5.8	5.6	5.7	5.9	5.6	5.7
Gain per 100 g of food	24.0	22.6	21.8	7.1	5.6	1.7	7.5	5.6

The results with one litter are presented in Table I and require little comment. The animals receiving cystine and glycine as supplements gained weight rapidly, an average weight increment of 22.8 g per 100 g of food consumed, while the animals which received dibenzoylcystine and glycine showed no better growth than the animals fed the basal diet supplemented with glycine. It is evident that under our experimental conditions, even though dietary glycine were available to detoxicate any benzoic acid which might be formed in the biological hydrolysis of the benzoyl derivative of cystine, this hydrolysis did not *proceed with sufficient rapidity* to permit dibenzoylcystine to serve as an *effective* source of cystine for the purposes of growth of the young white rat. The hydrolysis of dibenzoylcystine, if it occurs in the gastro-intestinal tract of the young white rat, is probably without any biological significance.

Summary. Young white rats were fed *l*-cystine and dibenzoyl-*l*-cystine as supplements to a basal diet known to be low in its content

[5] Curtius, T., and Kyriacou, N. C., *J. prakt. Chem.*, 1917, **95**, 360.

of cystine, in an attempt to determine whether dibenzoyl-*l*-cystine could be hydrolyzed in the organism and thus serve as a significant source of cystine for purposes of growth. To detoxicate any benzoic acid which might be formed in the hydrolysis and afford optimal conditions for observation of the growth-promoting effect of any cystine formed, glycine was also added to the diets. No evidence was obtained that dibenzoyl-*l*-cystine under the experimental conditions employed could serve as an effective source of cystine for purposes of growth.

10183

Effect of Methionine on Casein Metabolism.*

M. C. KIK. (Introduced by Barnett Sure.)

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Many investigators have reported that cystine stimulates growth in rats fed a ration containing casein as the sole protein. This ration is low in cystine. Mitchell,[1] Greaves and Morgan,[2] and Kik[3] have also shown that the biological value of casein, as determined by short metabolism experiments according to Mitchell's method, increased after addition of cystine.

Some years ago, Jackson and Block[4] produced evidence that methionine like cystine is capable of stimulating growth in rats fed a low cystine diet and quite recently Rose and coworkers[5] proved that methionine is an indispensable amino acid, 0.6% of which is needed in the diet for a normal increase in weight. According to them cystine is dispensable and stimulates growth only when methionine is present in too low a level.

In the present communication results are reported of a metabolism experiment with rats for determining the effect of methionine versus cystine addition on the biological value of casein for maintenance and growth, as evidenced by nitrogen balance experiments.

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* Research paper No. 481, Journal Series, University of Arkansas.

1 Mitchell, H. H., *J. Biol. Chem.*, 1924, **58**, 923.

2 Greaves, E. D., and Morgan, A. F., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 506.

3 Kik, M. C., *Ark. Agr. Exp. Sta. Bull.* 352, 1938.

4 Jackson, R. W., and Block, R. J., *J. Biol. Chem.*, 1932, **98**, 465.

5 Womack, M., Kemmerer, K. S., and Rose, W. C., *J. Biol. Chem.*, 1937, **121**, 403.

Six male rats, litter mates, 60-64 g in weight were employed for the determination of the biological value of casein fed at a 9% protein level (1.46% N in the ration). Mitchell's method was used. In the first period 3 rats were fed the casein ration, and the other 3 received the same ration and 25 mg cystine daily. In the second period all 6 rats were placed on the standardizing ration and in the third or last period 3 rats received the casein ration and the remaining 3 the same ration and 25 mg methionine daily. The results of this experiment are presented in Table I, whch shows that the average biological value for casein was 74.9; for casein and cystine 79.8 and for casein and methionine 84.0. The average true digestibility was 100, 99.3, and 100 respectively.

TABLE I.

Ration	Rat No.	Biological value, %	True digestibility, %	Increase in wt, g	Food consumption, g	Gain per g of food consumed, g	Avg
Casein	1	74.0	100.0	21	114.7	.183	
	3	70.0	100.0	30	111.8	.268	
	5	77.1	100.0	30	109.0	.267	.239
Casein and Cystine (25 mg daily)	2	81.3	97.8	31	110.0	.282	
	4	77.5	100.0	34	109.0	.312	
	6	80.7	100.0	33	105.7	.317	.303
Casein and Methionine (25 mg daily)	1	87.3	100.0	30	99.6	.301	
	3	81.4	100.0	31	99.0	.313	
	5	83.2	100.0	37	95.8	.386	.333
Casein	2	79.3	99.6	23	109.6	.209	
	4	73.5	100.0	22	105.1	.209	
	6	74.7	98.4	28	109.4	.256	.225

A study of the growth and food consumption records confirms this. The gain per gram of food consumed during the experimental period is the lowest for the casein ration, and the highest for the casein and methionine ration, while this gain is higher for the latter ration than for the casein and cystine ration.

This indicates the better utilization of food for the casein ration supplemented by methionine. With supplementation of 25 mg daily, the optimum amount of 0.6% methionine is very closely reached; the methionine content of the unsupplemented casein ration is about 0.34%. Casein contains approximately 3.2% methionine,[6] and there is 10.2% casein in the ration.

Summary. Methionine promotes the nitrogen retention of casein and in this experiment, proved even better than cystine.

6 Tucker, H. F., and Eckstein, H. C., *J. Biol. Chem.*, 1937, **121**, 479.

10184

On a Sex Difference of the Histamine Content of Blood of the Rat.

BRAM ROSE.* (Introduced by J. S. L. Browne.)

From the McGill University Clinic, Royal Victoria Hospital, Montreal, Canada.

During the course of recent investigations on the histamine content of the blood of the rat, it was noted that there was a difference in the control values obtained in female as compared with those obtained in male animals. The whole blood histamine content of 40 male and 34 female rats was studied and the average blood histimine content of the male group was found to be 0.035 gamma per cc (expressed as base) whereas that of the female group was 0.06 gamma per cc (Fig. 1). The animals were of the hooded strain, ranging in weight from 140 to 220 g, and all of the same colony.

The blood histamine was determined by the method of Barsoum and Gaddum[1] as modified by Code.[2] The assays were done on the isolated guinea-pig ileum suspended in Tyrode solution at 37°C.

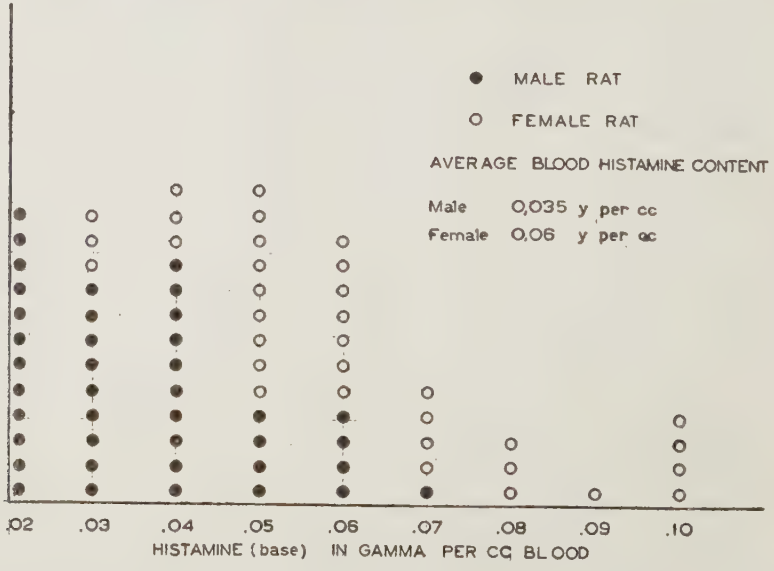

FIG. 1.

Showing the distribution of individual determinations in the male and female groups.

* Aided by a grant from the Banting Research Foundation, Toronto, Canada.

1 Barsoum and Gaddum, *J. Phys.*, 1935, **85**, 1.

2 Code, C. F., *J. Phys.*, 1937, **89**, 257.

The animal was anesthetized with ether and the sample of blood then removed from the inferior vena cava.

The significance of this difference has not yet been worked out although the effect of adrenalectomy in both male and female, and of ovariectomy in the female has been studied. Neither of these two procedures alter the resting blood histamine as obtained by this method. It is, however, interesting to note that the histamine content of the ovary is 10 times that of the testis (Gaddum).[3] Also that recently Ungar and Dubois[4] have shown the presence of a histamine-like substance in human pregnancy urine.

Conclusions. The histamine content of the whole blood of the normal female rat is higher than that of the normal male rat.

10185 P

Effect of Local Application of Testosterone in an Ointment on Growth of Penis in the Rat.*

R. R. GREENE AND H. S. WIGODSKY. (Introduced by A. C. Ivy.)

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The purpose of this experiment was to determine whether local application of testosterone to the penis in mammals would result in growth and development of this organ. This was suggested by the experimental evidence of absorption and systemic effects of androgens when applied to the skin[1] and the particularly intensified action of androgens on combs of chickens when applied locally.[2]

Twenty-four-day-old albino rats from our colony were used in these experiments. The treated and control animals in each group were arranged so that comparisons were made between litter mates. Each treated animal received 0.075 mg testosterone† daily by local application or subcutaneous injection for 22 days. The prepuce was drawn back and a measured amount of testosterone ointment or

3 Gaddum und Dale, *Gefässerweiternde Stoffe der Gewebe*, G. Thieme, 1936, p. 42.

4 Ungar, G., et Dubois, J., *C. R. de la Soc. de biol.*, 1937, **125**, 963.

* Supported in part by a grant from the Josiah Macy, Jr., Foundation.

1 Moore, C. R., Lamar, J. K., and Beck, N., *J. Am. Med. Assn.*, 1938, **111**, 11.

2 Fussgänger, R., *Med. Chem. Z.*, 1934, **2**, 194.

† We wish to thank Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products for the testosterone used in this work.

plain lanolin was applied to the penis daily from a tuberculin syringe. The testosterone ointment was prepared by dissolving crystalline synthetic testosterone in heated anhydrous lanolin at a concentration of 3.5 mg per cc. The testosterone administered by subcutaneous injection was dissolved in peanut oil. The controls in this group received an equivalent amount of plain peanut oil.

The animals were killed and examined at the age of 47 days, 23 days after treatment had begun. At autopsy the penis was dissected free from the prepuce and surrounding tissue, measured from its distal end to the bulbo-cavernosus muscles, excised and weighed. The prostatic-seminal vesicle complex was dissected free and weighed. In group II the testes were also weighed.

Group I consisted of 5 litters. Each litter was divided into (A) castrated animals treated with testosterone (0.075 mg daily) ointment, (B) castrated animals treated with plain lanolin, and (C) untreated intact control animals. In the latter, the prepuce was drawn back daily but no treatment was administered. The number of animals and results are tabulated. The penes of the testosterone treated animals averaged 31% longer and weighed 71% more than those of the lanolin treated castrates and were approximately equal to those of the intact animals.

Group II consisted of 5 litters, each divided into (A) intact animals treated with testosterone (0.075 mg daily) ointment and (B) intact animals treated with plain lanolin. The number of animals used and the results are tabulated. In these animals no appreciable differences in penile length or weight were obtained. The testes weighed slightly less and the prostatic-seminal vesicle complex slightly more in the treated animals than in the untreated animals, but the differences are probably not significant.

Group III consisted of 5 litters, each divided into (A) castrated rats injected subcutaneously (0.075 mg daily) with testosterone in peanut oil and (B) castrate rats injected subcutaneously with plain peanut oil. The number of animals used and the results are given in Table I. The penes of the treated animals were 21% longer and weighed 114% more than those of the controls. The prostatic-seminal vesicle complex of the treated animals weighed 113% more than those of the control castrates.

Further investigations are being carried out to determine the effect of small doses on a large series of animals.

Summary. 1. Seventy-five gamma of testosterone in an ointment applied locally to the penes of castrate rats for 22 days results in growth of the penis of approximately the same order as that in un-

TABLE I.

Effect of Local Application of Testosterone in an Ointment on Growth of the Penis in the Rat.

Group	Animals	No.	Treatment	Avg wt, g	Avg length of penis, cm	Avg wt of penis, mg	Avg wt of prostatic Sem. Ves., mg	Avg wt of testes, g	Avg % increase length of penis	Avg % increase wt of penis	Avg % increase prostatic Sem. Ves.
I A	Castrated	8	Local application testosterone in lanolin	93.7	2.1	80	106	—	31	71	116
I B	”	9	Lanolin only	87.5	1.6	45	49	—			
I C	Intact	6	No treatment	90.0	2.1	89	124	—	31	98	153
II A	”	11	Local application testosterone in lanolin	85.0	2.0	79	161	0.952	5	5	18
II B	”	10	Lanolin only	84.0	1.9	75	136	0.989			
III A	Castrated	9	Subcutaneous injections testosterone in peanut oil	87.4	1.9	73	113	—	21	114	113
III B	”	9	Subcutaneous injections peanut oil only	88.7	1.6	34	53				

treated normal animals or in castrated animals treated by daily subcutaneous injections of the same amount of testosterone. 2. In the dosage used, growth of the prostatic-seminal vesicle complex in rats treated by local application of testosterone to the penis is similar to that obtained by subcutaneous injection. 3. As measured by gross testicular weights, the testes are not appreciably inhibited by local application of testosterone to the penis, in the dosage used.

10186

Intravenous Injection of Amino Acids on Glucose Utilization Rate of Hypophysectomized and Insulin-Treated Rabbits.

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The high utilization rate[1] of the hypophysectomized rabbit makes it an excellent preparation for the study of the factors influencing carbohydrate metabolism. We attempted to determine if, and to what extent this sugar requirement could be substituted by amino acids. For this we carried out the determination of the glucose utilization rate in fasted hypophysectomized rabbits by the method previously described,[1] *i. e.,* by determining the rate at which glucose must be injected intravenously to maintain the blood sugar at a constant normal level. Having established this rate, we measured the effect of injecting a solution of amino acids,* containing 1.0% nitrogen (Frederick Stearns and Co.). In the first animal studied it was found that the glucose injection rate necessary to maintain a normal blood sugar level was markedly decreased during the injection period of the amino acids. We found the lowered glucose utilization period persisted for some 24 hours after the stoppage of

[1] Greeley, P. O., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1070.

* We wish to express our appreciation to the Frederick Stearns and Company Laboratories for the supplies of amino acids used in this work. We are indebted to Doctor Melville Sahyun, Director of Biochemical Research of that Company, for the constitution of the preparation. He states, "The amino acids are prepared by hydrolysis of casein in sulphuric acid and the subsequent removal of sulphate, ammonia, calcium and phosphate; also the separation of the non-hydrolyzable fraction by adsorption so that the final preparation consists of the pure amino acids from casein hydrolysate." The distribution of amino acids in the mixture is probably similar to that of hydrolyzed casein from which it was prepared.

amino acid injection. This could not be explained as a substitution and led us to the investigation of the effect of intravenously injected amino acids on glucose utilization rate.

Four hypophysectomized rabbits were used. First, the maximum glucose utilization rate was determined as described previously.[1] We were unable to explain the variation in this maximum rate in different rabbits. Then the glucose utilization rate was determined for the next period, about 2 hours in duration, during which the solution of amino acids was injected. These rabbits were observed up to 48 hours following this injection period. (Table I.)

TABLE I.
Hypophysectomized Rabbits.

Rabbit No.	Glucose per hr: Before injecting amino acids g	Glucose per hr: After injecting amino acids g	Amino acid solution injected cm^3	Hr followed after injecting amino acids	Glucose spared g	Glucose equivalent of amino acids injected g
1	1.23	0.360	77	25	21.8	3.9
J.	1.39	0.375	120	48	48.8	6.1
G.	0.284	0.10	37	48	8.8	1.87
F.	0.720	0.119	31	48	28.8	1.57

The results (Table I) show a marked effect of the amino acids on the glucose utilization. That this is not due to any large degree to substitution follows from a qualitative examination of the figures. In the sixth column is given the amount of glucose "spared" by the amino acid injection. That is the difference between the glucose which would have been used had the animal maintained the maximum rate (column 2) and the amount actually used (column 3) during the after period (column 5). These amounts of glucose are quite large and could not have come from the conversion of the amount of amino acids given, to glucose. The true glucose-nitrogen ratio for casein (the amino acids were essentially those of casein) has not been determined. The excretory G:N ratio for casein in the phloridzin dog[2] is 3.06. Drury, Bergman and Greeley[3] stated the "true" G:N ratio for body protein of the phloridzinized dog as 6.04 as compared to the "excretory"† G:N ratio of 3.65. If we assume

[2] Janney, N. W., *Biol. Chem.*, 1915, **20**, 321.

[3] Drury, D. R., Bergman, H. C., and Greeley, P. O., *Am. J. Physiol.*, 1936, **117**, 323.

† The classical G:N ratio (here referred to as "excretory") considers only that glucose which is excreted by the kidneys and does not include that glucose formed from protein which is oxidized by the tissues. The total or "true" G:N ratio includes both the glucose excreted and that oxidized by the tissues.

the same relationship to exist between "excretory" and "true" G:N ratios for casein we would have 5.07 g of glucose formed for each gram of protein nitrogen. In column 7 is given the possible glucose that might come from the amino acids figured from this ratio. Close calculation is not necessary, for one could assume that the entire amount of the amino acids be converted to glucose and still be unable to account for the change in glucose utilization of the rabbits in this way.

We conclude that this effect is not substitution from another observation. To 2 hypophysectomized rabbits, after attaining their maximal glucose utilization rate, we administered 40 cc amino acids by stomach and followed them thereafter. In one case the utilization rate before the giving of the amino acids was 390 mg per hour; for the 6 hours after the amino acid feeding the rate was 146 mg per hour and during the next 13 hours 308 per hour. The comparable figures for the other animal were 680 mg, 627 mg, and 500 mg. These results suggest very little if any of the specific action obtained by intravenous injection, and can rather be explained as a substitution effect. The gastro-intestinal tract should be the route by which maximal substitution should be effected since the amino acids after absorption are largely taken up by the liver and converted very rapidly to glucose. As shown by Van Slyke and Meyer[4] only a small fraction of the fed amino acids gets into the general circulation and to the other tissues.

Having established this effect in hypophysectomized rabbits we next attempted to determine whether amino acids had a similar effect on rabbits with a high glucose utilization rate from another cause, insulin.‡ Two male 3-day-fasted rabbits of the same weight (2 kg) were both given an injection of 2.5 units insulin intravenously and each hour thereafter 1 unit subcutaneously for 10 hours. One of these was given 40 cc amino acid solution (containing 2.5 g amino acids). Both were given just enough glucose intravenously to maintain them at a normal blood sugar level. At the end of 11 hours the control rabbit had required 11.3 g glucose whereas the amino-acid-injected rabbit had required 5.9 g. The difference, 5.4 g, could not all have come from amino acids since if we assumed even a 100% conversion of amino acids to glucose it would give only 2.5 g. This experiment was repeated on two 2-day fasted male rabbits of the same weight (2.1 kg). Everything was

[4] Van Slyke, D. D., and Meyer, G. M., *Biol. Chem.*, 1913, **16**, 231.

‡ We wish to express our appreciation to the Eli Lilly Company for the insulin used in these experiments and to the Abbott Laboratories for the nembutal used in this work.

carried out as in the previous experiment except that the hourly insulin was given I.V. and the experiment continued for 15 hours. At the end of this time the control had required 22.2 g sugar, the amino acid rabbit 11.4 g, a difference of 10.8 g glucose compared to 2.5 g amino acids injected.

There can be no question of the action of this preparation of amino acids on the glucose utilization rates of hypophysectomized, and of insulin-injected rabbits. We are continuing our work in the attempt to determine what constituents are responsible for this action. Jacobs[5] found that cysteine diminishes the hypoglycemic action of insulin and believes this to be due to a specific action of the sulphydryl group. Our results could hardly be explained on this basis since the mixture we used contained no cysteine and very little cystine (0.3 to 0.5% of the total amino acid content).

Summary. A mixture of amino acids when injected intravenously into rabbits with high glucose utilization rates (from hypophysectomy, and from insulin) markedly reduced this glucose requirement.

10187

Effect of Vitamin B_1 and Vitamin B_2 Complex on the Loss of Weight Produced in Rats by Experimental Hyperthyroidism.

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Himwich, Goldfarb and Cowgill[1] reported that an increased amount of undifferentiated vitamin B_1 is needed during experimental hyperthyroidism, and Sure and Buchanan[2] found that vitamin B_1 has an antithyrogenic action. Later Drill[3] found that a large amount of yeast fed to rats receiving thyroxin will prevent a loss of liver glycogen. In this investigation a study was made on the effect of vitamin B_1 and of a yeast concentrate on the loss of weight produced by experimental hyperthyroidism.

Adult rats, weighing about 250 g, were all fed diet No. 8 *ad libitum*. This diet consisted of: salts, 4; cod liver oil, 4; Crisco,

5 Jacobs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 305.

1 Himwich, H. E., Goldfarb, W., and Cowgill, G. R., *Am. J. Physiol.*, 1932, **99**, 689.

2 Sure, B., and Buchanan, K. S., *J. Nutrition*, 1937, **13**, 513.

3 Drill, V. A., *J. Nutrition*, 1937, **14**, 355.

10; casein, 20; and cornstarch, 62 parts. Dried yeast (200 mg) was fed to each rat per day. This supplied 3.6 international units of vitamin B_1 and 4 Sherman-Borquin units of vitamin G (flavin) per day.

EXP. 1. Female rats were used in this experiment. Normal controls (5 rats), receiving diet No. 8 *ad libitum*, made a constant gain in weight. The thyroid fed controls (5 rats), received diet No. 8 *ad libitum* plus 100 mg of thyroid gland per day. The thyroid-fed controls were used to show that the amount of thyroid gland fed was sufficient to produce a continued loss in the weight of rats receiving a normal diet (No. 8). These rats showed a rapid decline in weight. Twelve test rats were also fed diet No. 8 plus 100 mg of thyroid gland per day for 17 days, during which time they lost an average of 23 g in weight. (See Table I for the average weight change of the rats.) On the seventeenth day a daily injection of 500 gamma of vitamin B_1 was begun in the test rats while they were still receiving thyroid gland. The female hyperthyroid rats injected with vitamin B_1 stopped losing weight, while the thyroid-fed controls, receiving thyroid gland but no injections of vitamin B_1, continued their rapid loss of weght. Although the test rats did not lose any more weight when injected with vitamin B_1, they did not regain any of their lost weight. On the thirtieth day of the experiment 5% of a brewer's yeast concentrate was included in diet No. 8 and fed to the test rats. An immediate gain in weight, as is shown in Table I, was then made. The rats continued to gain weight, while still receiving thyroid gland, up to the eighty-first day, at which time they were dissected for tissue studies. This experiment shows that vitamin B_1 and yeast concentrate, a rich source of the vitamin B_2 complex, enabled the test rats to regain their lost weight while thyroid gland was being fed. Vitamin B_1 alone did not enable the test rats to regain their lost weight.

EXP. 2. Male rats were used in this experiment. The diets, dosages, days of feeding and the number of rats were the *same* as in Experiment 1. As will be seen from Table I, the normal controls gained in weight, the thyroid-fed controls lost weight, and the test rats also lost weight and did not regain their lost weight when treated with vitamin B_1 and a yeast concentrate. Thus, a sex difference in response is present. The vitamin B_1 and yeast concentrate treatment enabled the female hyperthyroid rats to regain the amount of weight lost, while they were still receiving thyroid gland, whereas the male rats on the same dosage of thyroid gland did not recover their lost weight when treated.

TABLE I.
Average Gain or Loss of Weight of the Rats in Grams.

Days of Exp.	Experiment 1			Experiment 2			Days of Exp.	Experiment 3		
	Normal Controls	Thyroid-fed Controls	Test Rats	Normal Controls	Thyroid-fed Controls	Test Rats		Normal Controls	Thyroid-fed Controls	Test Rats
5	— 1g	—25	—21	+ 2	— 19	—20	5	+ 7	—28	—15
12	+ 5	—28	—21	+10	— 22	—25	10	+ 9	—29	—24
16	+10	—22	—20	+14	— 20	—29	14	+10	—34	—36*
17	+ 6	—25	—23*	—	— 30	—29*	17	+19	—38	—31
19	+ 5	—26	—17	+30	— 32	—21	21	+24	—56	—35
23	+ 5	—27	—22	+25	— 50	—24	24	+27	—67	—29
26	+ 5	—41	—21	—	— 74	—26	31	+26	—57	—31
30	+10	—59	—15†	+22	—102	—15†	35	+35	—69	—32
39	+17	—67	—10	+32	—122	—40	38	+37	—82	—31†
45	+22	—74	— 7	+31	—137	—50	42	+37	‡	—24
51	—	—86	— 6	—	‡	—	45	+40		—21
57	+24	‡	— 1	+26		—45	52	+45		—12
60	+33		— 6			‡	56	+44		+ 4
64	+29		— 4				58	+47		+ 6
70	+25		+ 1							
81	+32		+ 4							

*Daily subcutaneous injections of 500 gamma of vitamin B_1 begun.
†5% yeast concentrate added to diet No. 8 and fed to test rats in addition to the daily injection of 500 gamma of vitamin B_1.
‡The remaining rats were chloroformed and dissected.

EXP. 3. Male rats were used in this experiment. The same number of rats were used in each group as in Experiment 1. In this experiment the rats were fed 50 mg of thyroid gland per day, which is one-half of the former dosage. As will be seen from Table I, the normal controls, receiving only diet No. 8, gained in weight. The thyroid-fed controls, fed diet No. 8 plus 50 mg of thyroid gland per day, rapidly lost weight. The test rats also received diet No. 8 plus 50 mg of thyroid gland per day. These rats also lost weight. On the fourteenth day of thyroid feeding the daily injection of 500 gamma of vitamin B_1 was begun in the test rats. The injected rats stopped losing weight, but did not regain any of their lost weight until, as in Experiment 1, 5% of a brewer's yeast concentrate was included in diet No. 8 and fed to the test rats. The yeast concentrate was given to the test rats on the thirty-eighth day of the experiment, and a constant gain in weight was then made. Thus, male rats, which would not respond to vitamin B_1 and yeast concentrate treatment when receiving 100 mg of thyroid gland per day, responded when the dosage of thyroid gland was 50 mg per day.

When hyperthyroid rats are restricted to 12 g of food per day, they do not stop losing weight when vitamin B_1 is injected, even though there is no subnormal amount of vitamin B_1 in the tissues.[4] Thus caloric intake is an important factor in studying the gain of weight during experimental hyperthyroidism. This will be discussed at length elsewhere.

Conclusions. 1. Hyperthyroid rats which had lost weight and were still receiving thyroid gland regained their lost weight when *both* vitamin B_1 and the vitamin B_2 complex were administered. 2. A sex difference in response is present.

[4] Drill, V. A., *Am. J. Physiol.*, 1938, **122**, 486.

10188 P

Quantitative Enzymic Conversion of Cocarboxylase (Vitamin B_1-Pyrophosphate) to Free Thiamin.

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Recently Lohmann and Schuster[1] reported that thiamin (vitamin B_1) may exist in nature as a pyrophosphoric acid ester to constitute cocarboxylase, the coenzyme of carboxylase. Thiamin itself cannot function as the coenzyme, although the action of pure cocarboxylase *in vitro* is strongly stimulated by the addition of the pure vitamin.[2] However, pigeon curative tests have shown that cocarboxylase is practically as equally effective as free thiamin. A number of workers[3-6] have subsequently reported that in the thiochrome method for determining thiamin the cocarboxylase forms a thiochrome but this is not extracted by isobutanol and hence cannot be estimated.[4] For this reason analyses by this method of preparations rich in cocarboxylase (such as yeast), give values decidedly less than those obtained by biological assay.[6] Cocarboxylase may be hydrolyzed[1] to the monophosphoric acid ester of thiamin by 1 N HCl solution, but the latter ester is resistant to acid hydrolysis. Alkaline hydrolysis will completely remove the second phosphoric acid grouping but inasmuch as there is a concomitant destruction of the end-product (thiamin) such a procedure is impractical for analytical purposes. Levine[6] has observed that, when aqueous yeast extracts are allowed to stand at room temperature exposed to the atmosphere for a period of 48 hours with no preservative added, there is a conversion of the cocarboxylase present to free thiamin. He attributed this change to the activity of a yeast phosphatase. Kidney phosphatase[1, 5, 7] has been used as means for hydrolyzing the coenzyme to the free vitamin. However, with such a preparation the conversion does not appear to be quantitative.

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* Upjohn Fellow in Clinical Research, 1937-1939.

1 Lohmann, K., and Schuster, P., *Biochem. Z.*, 1937, **294**, 188.

2 Ochoa, S., *Nature*, 1938, **141**, 831.

3 Kinnersley, H. W., and Peters, R. A., *Biochem. J.*, 1938, **32**, 677.

4 Roth, H., *Biochem. Z.*, 1938, **297**, 52.

5 Cerecedo, L. R., and Hennessy, D. J., *Abstr. Am. Chem. Soc., Div. Biol. Chem.*, September, 1938.

6 Levine, H., unpublished data.

7 Tauber, H., *J. Biol. Chem.*, 1938, **123**, 499.

In the present study an extension of our chemical method[8] for the estimation of thiamin in biological materials has been applied to an investigation of the conversion of cocarboxylase to thiamin. We have found that both cocarboxylase and the monophosphoric acid ester of thiamin† react with diazotized p-amino acetophenone to yield colored solutions but that these pigments are not extracted by xylene. This is comparable to the findings recorded with the thiochrome method for determining thiamin. Tests conducted on a number of yeast extracts have not indicated that bacteria play a part in the conversion of cocarboxylase to thiamin, but that this change results from the action of a phosphatase which is liberated from the cell during the drying of the yeast. Thus, aqueous solutions obtained by initial alcoholic extraction of yeast or aqueous extraction at 70°C contain cocarboxylase in amounts comparable to that originally present in the yeast powder, and these amounts are not changed by subsequent bacterial contamination. The aqueous extraction of the yeast powder *at room temperature* gives solutions which will completely hydrolyze not only its own cocarboxylase but also added phosphorylated thiamin. The incubation of an aqueous suspension of the whole yeast powder under aseptic conditions, with toluene as a preservative, results in a complete hydrolysis of the cocarboxylase present. The free thiamin may then be quantitatively extracted by heating one-half hour at 70°C at a slightly acid pH in an atmosphere of nitrogen and removed from the insoluble residue by centrifugation. Chloroform completely prevents the conversion of cocarboxylase to free thiamin.

The pH optimum for the hydrolysis of cocarboxylase is rather closely restricted to that of 4.5. The enzyme is very active over a wide range of temperature, from 10 to 55°C, with the optimum at 45°C. At 70°C the enzyme activity is irreversibly destroyed. Under the optimal conditions given the reaction is complete in 12 hours. Yeast preparations or extracts, devoid of soluble protein, have not been found to show enzyme activity. Methods for the isolation of the undenatured protein fraction of the active yeast powder have yielded products free of thiamin but still capable of hydrolyzing, though not completely, the phosphorylated vitamin extracted from the yeast powder and also the synthetic monophosphoric acid ester.

The incubation of our active yeast powder with a number of

[8] Melnick, D., and Field, H., Jr., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 723.

† We are indebted to Drs. Joseph L. Melnick and Kurt G. Stern, of the Laboratory of Physiological Chemistry, Yale University, for a generous supply of the synthetic crystalline monophosphoric acid ester of thiamin.

vitamin B_1 concentrates, such as extracts of yeast, rice polish and wheat germ, has enabled us to convert all of the vitamin present into the form by which it may be determined chemically. By difference between the thiamin values obtained before and after hydrolysis, the concentration of the vitamin in the phosphorylated form may be estimated. In the samples assayed we have found some yeast preparations to contain as much as 75% of its thiamin in this form, a rice polish extract 17%, and a wheat germ preparation 10%. Obviously, in the preparation of any dried yeast powder or extract the ratio of phosphorylated to free thiamin in the final product is dependent upon the conditions associated with the preparations. If during the process conditions are such as to allow the phosphatase present to act, a much smaller ratio will be obtained. Our findings of an autohydrolysis of the phosphorylated thiamin in aqueous suspensions of dried yeast powders support the earlier findings of Auhagen[9] that autolyzed yeast loses its cocarboxylase activity.

10189

Cortical Hormone-Like Action of Progesterone and Non-Effect of Sex Hormones on "Water Intoxication."*

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Gaunt and Hays have shown that crystalline progesterone maintains life and excellent health in adrenalectomized ferrets, whereas the estrogens are toxic and testosterone non-beneficial.[1] These findings probably account for the survival of pseudopregnant adrenalectomized animals.[2] † To determine if other species would respond

[9] Auhagen, E., *Biochem. Z.*, 1933, **258**, 330.

* This work was aided by a grant from the Penrose Fund of the American Philosophical Society.

The authors are indebted to Dr. Erwin Schwenk, of the Schering Corporation, for the progesterone and testosterone propionate used here; to Dr. Oliver Kamm, of Parke, Davis & Co., for the cortical hormone and theelin; and to Dr. J. A. Morrell, of E. R. Squibb & Sons, for the Amniotin.

[1] Gaunt, R., and Hays, H. W., *Science*, in press.

[2] Literature cited by Gaunt, R., and Hays, H. W., *Am. J. Physiol.*, in press.

† Dr. G. W. Thorn reports a confirmation of the life-maintaining action of progesterone in adrenalectomized dogs (personal communication).

similarly, we have tested the effects of progesterone on the life-span of 30-day-old adrenalectomized rats.

Progesterone in oil was given in doses of 1-2 mg per day for 20 days, the dose varying somewhat with the apparent needs of the animal. Untreated littermates were used for controls. Results are shown in Table I. Series I was run during hot summer months (at C.S.H.) and the short survival of controls was due to that fact. Series II was run under our usual, more optimal, conditions for observation of adrenalectomized rats (at W.S.C.). Progesterone pellets implanted under the skin at the base of the ear were not noticeably beneficial. In half of the cases life was maintained as long as progesterone was given, and a definite life-extension was seen in those animals which died on treatment. Equally marked was the greater weight gain and growth in treated rats.

The fact that all animals could not be maintained for the duration of treatment was probably due to inadequate dosage, but difficulties in acquiring large amounts of progesterone prevented tests at higher levels. The dose necessary for the maintenance of a 50 g rat is about the same as that needed for a 700-1000 g ferret. This need for high dosage in the rat probably accounts for the many previous failures to notice the cortical hormone-like action of the luteal hormone[2]

Water Intoxication Studies. The sex hormones and the adrenal cortical hormones affect electrolyte and water metabolism of intact animals somewhat similarly (Thorn and Harrop and others[3, 4, 2]). One manifestation of an irregular electrolyte and water metabolism in adrenalectomized animals lies in their sensitivity to excess water, to which they react by the rapid appearance of "water intoxication" symptoms and death.[5, 6] These symptoms can be prevented by cortical hormone or salt.[5]

In view of the above facts we thought that some of the sex hormones, particularly progesterone, would probably modify the response to excess water, at least in adrenalectomized animals. Surprisingly, they had no effect either in normal or adrenalectomized rats, as seen from Table II. Experiments were conducted as previously described.[6] The rate of excretion of the water which had been administered by stomach tube, and the clinical symptoms were

[3] Thorn, G. W., and Harrop, G. A., *Science*, 1937, **86**, 40.

[4] Thorn, G. W., and Engel, L. L., *J. Exp. Med.*, 1938, **68**, 299.

[5] Swingle, W. W., Parkins, W. M., Taylor, A. R., and Hays, H. W., *Am. J. Physiol.*, 1937, **119**, 557.

[6] Gaunt, R., Remington, J. W., and Schweizer, M., *Am. J. Physiol.*, 1937, **120**, 532.

TABLE I.

Survival of Progesterone-treated, Adrenalectomized Rats.

	No. rats used	No. died during treatment	Avg survival and range of rats dying on treatment	No. lived throughout treatment	Avg survival after treatment stopped	Avg max. wt gain	Avg wt diff. between operation and death	No. surviving indefinitely
			Series I.					
			Days		Days	g	g	
Progesterone-treated	12	7	9.7 (3-18)	5	7	17.7	+11.6	1
Controls untreated	14	14	6.0 (3-20)	—	—	3.4	+ 0.4	0
			Series II.					
Progesterone-treated	7	3	12.0 (9-16)	4	16	23.1	+20.0	0
Controls untreated	8	8	10.0 (8-15)	—	—	5.0	+ 3.0	0

TABLE II.

Response to Excess Doses of Water.

No. Rats used	Treatment	Water dosage	Avg % H_2O excreted at 7 hr	Avg % H_2O excreted at 11 hr	No. convulsive or prostrated	No. dying in 24 hr
6	Intact—Untreated	.06 cc/g* x 6 hr	84.8	92.9	0	0
6	Intact—3,333 I.U. amniotin-in-oil	,,	68.4	89.3	1	0
12	Intact—Untreated	.07 cc/g x 6 hr	71.5	83.5	4	1
6	Intact—3,333 I.U. amniotin-in-oil	,,	61.3	83.6	0	0
19	Intact—400 I.U. theelin-in-H_2O	,,	70.7	90.3	0	0
14	Adrex.†—Untreated	.06 cc/g x 5 hr	30.0	37.5	11	11
19	Adrex.—433 I.U. theelin-in-H_2O	,,	22.5	34.7	9	9
9	Adrex.—5 mg testosterone in propylene glycol	,,	15.0	20.7	16	16
6	Intact—Propylene glycol (1.0 cc)	.07 cc/g x 6 hr	11.5	16.0	7	6
11	Adrex.—Propylene glycol (2.0 cc)	.06 cc/g x 5 hr	44.4	65.9	2	0
6	Adrex.—Progesterone (7.5 mg)	,,	36.4	39.0	8	7
6	Adrex.—Cortical extract (4.0 cc)	,,	80.0	89.8	2	0
6	Adrex.—Normal saline (4.0 cc)	,,	35.5	48.5	1	1

*Six hourly doses of 0.06 cc H_2O per g body wt.

†Adrenalectomized.

the criteria observed. Hormones in oil (Amniotin, progesterone) were injected in divided doses, the first 36 hours before the experiment was begun, and the last 1-5 hours before its beginning. Injections of theelin-in-water and testosterone in propylene glycol were started 12-18 hours before the test and the last injections given during the first hours of water administration. In one experiment 5 mg of crystalline progesterone was flushed into the peritoneal cavity in saline suspension 12 hours prior to the experiment, in addition to injections of the hormone in oil. Cortical hormone was given an hour before and during the experiment. The cortical extract used was made up in normal saline, but its effects were largely due to the hormone and not the salt as seen by comparison of hormone and saline treated groups. Adrenalectomy was done 12 hours before the experiments started.

As seen from Table II none of the hormones given, except cortical hormone, modified the response in any significant way from that of comparable controls. Propylene glycol was a slightly deleterious agent, but testosterone dissolved in it did not vary its effect. The characteristic inability of the adrenalectomized rat to eliminate excess quantities of water in the absence of cortical hormone or salt was in no way compensated for by the sex hormones. This was particularly surprising for progesterone, since this substance will keep most adrenalectomized animals alive, and indicates either a qualitative difference between cortical extract and corpus luteum hormone action, or at least a difference in the rate of that action. It is possible that progesterone must be converted into some other chemical form to exhibit cortical properties.

Summary. Progesterone in doses of 1-2 mg per day extended the lives of young adrenalectomized rats in half of the cases throughout a 20-day treatment period. On the basis of equivalent dosage, it was much less effective than in adrenalectomized ferrets. Unlike cortical hormone, neither progesterone, estrin nor testosterone modified the response of adrenalectomized rats to excess water.

10190 P

Mitotic Rhythm in Human Epidermis.

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Studies of mitotic rhythm, *per se,* have been few, although the number of mitoses found in various tissues has been used as an index of functional activity of these organs when subjected to various experimental conditions. The work of Loeb and his students[1, 2] on the thyroid gland, using the mitotic index as a basis for the measurement of the effect of various chemicals and hormones upon this organ is most significant. All the work that has been done on plants,[3–6] mice and cats,[7, 8] indicates the existence of a rhythm in cell division, which must obviously be taken into consideration in experimental studies; but, to the best of our knowledge, no observations have been reported on rhythmic periodicity of mitotic division in human tissues.

The relatively small number of mitoses that can usually be found in sections of human epidermis is striking, especially since the epidermis is known to be constantly renewed at a rather rapid rate, but as a general rule the sections of skin in which mitosis has been studied have been secured (by biopsy or by operation) during the day time. Consequently, the question arises whether more mitoses might not be observed in skin obtained during the night when, perhaps, cell division is occurring at a different rate than during the day.

The skin covering the prepuce of infants, which is removed at circumcision, was selected as a convenient tissue for experimentation. The babies were all of approximately the same age (8 days) and had been kept from birth under practically identical environmental

1 Friedman, H., and Loeb, L., *Anatom. Rec.*, 1934, **59**, 5.

2 Margolin, E. S., Proc. Soc. Exp. Biol. and Med., 1933, **30**, 495.

3 Karsten, G., *Z. f. Bot.*, 1915, **7**, and 1918, **10** (quoted by Carleton).

4 Stålfelt, M. G., *Kunst. Svenska Vetensk. Hand.*, 1921 **62**, 1 (quoted by Carleton).

5 Kellicott, W., *Bull. Torrey Bot. Club*, 1904, **31** (quoted by Carleton).

6 Friesner, R. C., *Am. J. Bot.*, 1920, **7**, 380.

7 Fortunyn-Van Leyden, Droogleever (Mrs.), *Proc. Soc. of Sciences*, Amsterdam, 1916, **19**, 38, and 1926, **29**, 979 (quoted by Carleton).

8 Carleton, A., *J. Anat.*, 1933, **68**, 251.

conditions, at the St. Louis Maternity Hospital. The specimens were obtained at various hours throughout the day and night, through the courtesy of the Director of the Hospital, Dr. Otto Schwartz. Immediately after circumcision (performed always by the same method), they were placed in a 1% solution of acetic acid and allowed to remain in this fluid for 24 hours. It was then possible to separate completely the layer of epidermis from the underlying dermis. This separated layer of epithelium was then stained *in toto* with Ehrlich's hematoxylin, dehydrated in alcohol, cleared in oil of wintergreen and xylol, and mounted. Mitoses were counted by using an oil immersion lens and focusing through the various cell layers, with less chance of missing cells in division than would be possible in serial sections. In each specimen 5000 cells were counted in successive fields and the number of cells in mitosis recorded. All counts were made by one observer in order to eliminate the element of individual variations in accuracy. Thus, the experimental error was reduced to a minimum.

In the specimens in which cell counts have been completed, a marked increase in the number of mitoses in the specimens obtained at night has been found.

TABLE I.

Time of taking specimen	No. of mitoses found	No. of cells counted
7:30 A.M.	14	5046
7:30 "	16	5034
9:00 "	10	5152
9:30 "	15	5014
10:25 "	7	5000
10:30 "	8	5068
11:40 "	28	5094
8:45 P.M.	23	5009
9:45 "	35	5124
10:45 "	31	5080
11:55 "	25	5013
11:50 "	23	5086
12:45 "	23	5016

Since the results obtained thus far seem to indicate that a rhythmical periodicity in mitotic division is present in human tissues, it is proposed to determine whether this rhythm is also present in cancer tissue, or whether it is altered or interrupted. In preparation for this study, carefully controlled biopsies are being made on tissue from patients with normal uterine cervices and from those with carcinoma of the cervix. One biopsy is obtained during the day and another at night from the same patient. The results of the mitotic counts upon this material will be reported later.

10191

Effect of Riboflavin and Thiamin Chloride upon the Cataractogenic Action of Galactose.*

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From Massachusetts State College, Amherst, Mass.

In previous work with galactose cataract the usual protective doses of vitamin supplements were provided in accordance with the recognized need of the rat. The normal growth of the rats and the lack of gross evidence of any vitamin deficiency led us to conclude that the cataract was probably due to some metabolic disturbance unrelated to a vitamin. The possibility of an increased demand for certain factors was considered, however. The usual dose of 0.5 g daily of dry yeast was increased fourfold without changing the rate or incidence of cataract. Massive doses of cod liver oil and viosterol made no difference.

The subsequent isolation of pure crystalline vitamins made it possible to supplement our previous findings by feeding massive doses of certain ones which have been shown to have any connection with lenticular changes. A previous paper reported[1] that no change in susceptibility to cataract was observed when large doses of ascorbic acid were administered orally or subcutaneously.

The present investigation using 2 crystalline fractions of the vitamin B complex, riboflavin and thiamin chloride (Betabion) has been made possible through the courtesy of Merck and Company. While no gross symptoms of vitamin B or G deficiency existed, nevertheless, it was possible that the introduction of galactose might have created unusual metabolic demands for one or both of these vitamins which could be recognized only by trying massive doses. So far as is known there is no etiologic factor common to galactose cataract and the vitamin G deficiency cataract first reported by Day, *et al.*[2] In fact, the statement made in a recent article by Day[3] "that flavin is *the cataract-preventive vitamin*" may need modifying in view of the present findings.

Four litters (29 rats) were so divided as to allow littermate comparisons and were fed a ration consisting of 25% galactose, 45% cornstarch, 15% vitamin-free casein, 4% salt mixture, 9% Crisco

* Contribution No. 323 of the Massachusetts Agricultural Experiment Station.

1 Mitchell, H. S., and Cook, G. M., *Arch. Ophth.*, 1938, **19**, 22.

2 Day, P. L., Langston, W. C., and O'Brien, C. S., *Am. J. Ophth.*, 1931, **14**, 1005.

3 Day, P. L., Darby, W. J., and Cosgrove, K. W., *J. Nutr.*, 1938, **15**, 83.

and 2% cod liver oil supplemented as indicated in Table I. Two drops of tikitiki were added to the crystalline supplements to provide the filtrate factor and incidentally to improve the palatability and thus insure complete consumption of the supplement. The doses of 50 micrograms of riboflavin and 20 micrograms of thiamin chloride were chosen as liberal allowances for the rat. The 0.5 g daily of dry yeast fed to the controls furnished 12-17 micrograms of thiamin and 30-50 micrograms of riboflavin.† Growth was normal in all groups except the one deprived of riboflavin

TABLE I.
Effect of Riboflavin and Thiamin Chloride upon Cataractogenic Action of a 25% Galactose Ration.

Ration supplement micrograms per day		No. of rats	Gain in wt in 3 weeks, g	Mature cataract development		
Riboflavin	Thiamin Cl			No. of eyes	days	Incidence, %
0	20	4	26.8	7	25.0	88
50	20	7	50.3	12	27.0	86
200	20	4	50.0	8	22.6	100
2000	20	5	59.0	8	27.0	80
50	2000	4	45.5	8	27.0	100
0.5 g yeast daily (control)		5	61.8	9	20.6	90

Cataract developed rapidly in all groups with no significant differences in rate or incidence. A few eyes invariably show resistance to advanced changes and this experiment was no exception. The last column in the table gives percentage of incidence of mature cataract which was high if not 100% in all cases. The average of 20.6 days required for cataract to develop in rats on the control ration in this experiment was somewhat shorter than previous averages (22.0 and 22.6 days) for other rats from the same colony on the same ration. Small differences in time and incidence cannot be considered significant. It would be erroneous to conclude that susceptibility to cataract was greater in rats receiving 200 micrograms of riboflavin than in those receiving 0, 50, or 2,000 micrograms because the incidence was slightly higher and the time a few days less. The one group receiving 2,000 micrograms of thiamin chloride required the longest time for cataract development but showed 100% incidence. None of these differences are significant statistically.

It may be concluded from these data that massive doses of riboflavin or of thiamin chloride exert no protective action against the development of galactose cataract in rats.

† Figures furnished by Fleischmann Laboratories.

10192

Blood Pressure and Hematology in Dogs Injected with Ant. Pituitary Extract.

F. C. Dohan, W. A. Jeffers and A. J. Creskoff. (Introduced by F. D. W. Lukens.)

From the George S. Cox Medical Research Institute, University of Pennsylvania, and the Robinette Foundation and the Hematology Section of the Medical Clinic, Hospital of the University of Pennsylvania, Philadelphia.

It has been observed that hypertension and polycythemia are frequently associated with pituitary basophilism. The effects of experimental hypophysectomy also suggest that the anterior pituitary may regulate blood pressure[1] and the number of erythrocytes.[2]

Because of these considerations a study was made of the effect of an extract of bovine anterior pituitary in 4 dogs. Dog No. 4 was

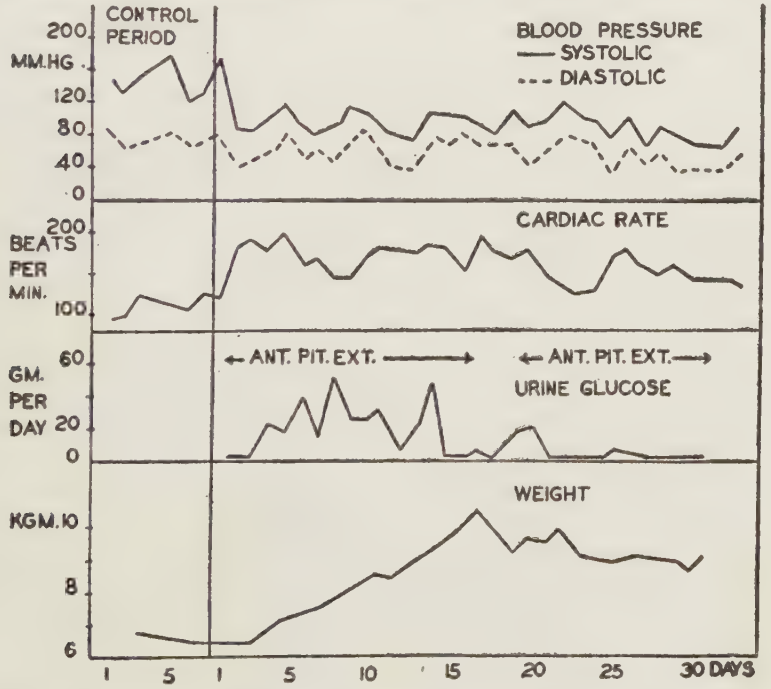

Fig. 1.

Dog 1. Illustrates the effect of increasing doses of anterior pituitary extract.

1 Houssay, B. A., *N. Eng. J. Med.*, 1936, **214**, 1086.

2 Stewart, G. E., Greep, R. O., and Meyer, O. O., Proc. Soc. Exp. Biol. and Med., 1935, **33**, 112.

TABLE I.
Erythrocyte and Platelet Data

	Dog No. 1			Dog No. 2			Dog No. 3			Dog No. 4	
Day of injection	0	8	18	0	7	*	0	10	18	0	6
RBC (millions)	7.4	6.0	4.4	6.5	6.1	5.0	8.1	6.7	6.7	5.8	5.0
Hgb† (%)	105	94	73	93	97	88	111	102	104	72	69
H'crit‡	54	45	33	47	46	36	56	44	45	35	30
Retics. (%)	0.1	0.3	0.7	0.2	0.1	0.4	0.1	0.1	0.1	0.2	2.0
Plate. (thousands)	280	360	260	270	340	310	300	240	290	290	210

*4th day after stopping injections.
†Sahli; 100% = 15.6 g/100 cc.
‡Van Allen Tube.

a male; the others were females. In general, the methods and technic of Young[3] were employed. With a few exceptions the injections were made daily into the peritoneal cavity. In general the dose was gradually increased from an amount of extract representing 10 g to an amount representing 30 g of gland. Dogs Nos. 1-4 were injected over periods of 31, 14, 26, and 8 days respectively.

The diet consisted of weighed portions of trimmed beef heart or a stock mixture of ground meat, cracker meal, chopped cabbage, cod liver oil and iodized salt. The blood pressure was determined daily in dogs No. 1 and 2 by the Collins method,[4] the animals having been trained to lie quietly. Hematological estimations were performed in duplicate on all 4 dogs.

The potency of the extract was illustrated by the marked glycosuria and weight gain (Fig. 1). These animals also developed polydipsia, insulin resistance, and diabetic glucose tolerance, lactation, and evidence of estrus, as well as retention of nitrogen such as is associated with the growth effect of anterior pituitary.[5]

In marked contrast to these metabolic effects were the slight changes in the blood pressure and blood picture. Both dog No. 1 and No. 2 showed an increase in heart rate (presumably a thyrotropic effect) which was accompanied by a slight fall in blood pressure (Fig. 1). The hematological findings were contrary to suggestions in the literature that an excess of anterior pituitary hormone may induce polycythemia. All 4 dogs showed reduced erythrocyte, hemoglobin and hematocrit values, while the number and immaturity of the leucocytes increased, reticulocyte and platelet numbers were not significantly altered (Table I).

The effect upon the blood pressure and blood picture in our animals treated with whole anteror pituitary extract is similar to that described by Thompson and Cushing[6] in a dog injected for 90 days with a gonadotropic fraction from sheep pituitaries.

Summary. No increase in blood pressure or in erythrocytes, reticulocytes, or hemoglobin was produced by repeated injections of an active crude saline extract of anterior pituitary.

3 Young, F. G., *Biochem. J.*, 1938, **32**, 513.

4 Collins, D. A., *Am. J. Physiol.*, 1936, **116**, 616.

5 Gaebler, O. H., and Price, W. H., *J. Biol. Chem.*, 1937, **121**, 497.

6 Thompson, K. W., and Cushing, H., *Proc. Roy. Soc. (B)*, 1934, **115**, 88.

10193

Non-effect of Irradiation of the Hypophysis in Sterile Monkey Females.

Carl G. Hartman and Charles Smith.

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Clinical literature contains numerous references to "cures" of sterility in women by means of "stimulating" doses of X-rays. Since the Carnegie Colony of rhesus monkeys has always included among its mature females a considerable percentage that were sterile because of failure to ovulate, the opportunity presented itself to put the theory of X-ray stimulation of the hypophysis to a critical test.

Thirteen animals were treated. In all treatments the following factors were constant: Kilovolts peak, 200; milliamperes, 20; target-skin distance, 50 cm; size of field, 4x5 cm; filters, ½ mm Cu + 1 mm Al; half layer value for ½ mm Cu = 1 mm Cu. The only variable was the r unit. The dosage was applied at one sitting with the animal under light nembutal anesthesia.

Pertinent data concerning the experiments are condensed in Table I.

Only animals in good general physical condition at the time of the experiments were used. It should be stated, however, No. 439 died in March, No. 489 in May.

Except for the control, No. 381, which was a regular ovulator both before and after the experimental cycle, only such animals were selected as failed to ovulate for the 2 cycles just preceding the experiment. Nos. 402, 435, and 441, however, experienced several ovulatory cycles earlier in the season.

Estimation of size of ovaries and uterus was made by palpation. While this method leaves an element of doubt in borderline cases, in the present series the diagnoses were unequivocal.

From the table it is seen that in all cases with a dosage of 60 to 160 r the effect was negative, that is, the animals that were not ovulating before radiation remained refractory and did not ovulate during the remainder of the season. Three of these animals, however, recovered full reproductive activity the following autumn, ovulating spontaneously and conceiving. Several times it was thought that the uterus had been stimulated slightly but of this one could not be certain.

With a dosage of 320 r one of 2 animals ovulated. It thus

TABLE I.

1. No.	381*	424*	428	435	439	440*	453*	489	460*	466*	402*	433*	441*
2. Wt	3640	4090	3520	3750	4750	4350	3500	5800	4330	4470	4170	3850	4090
3. Date, 1937	1/23	1/23	1/23	1/23	1/23	1/23	1/23	1/23	2/6	2/6	3/10	3/10	3/10
4. Dosage (r)	120	120	120	80	60	120	160	160	320	320	400	400	400
5. Day of Cycle	4	6	6	3	60	6	1	10	17	11	9	12	10
6. Prior Cycles (days)	28+	53—	124—	31—	28—	25—	53—	30—	61—	32—	48—	24—	33—
	23+	42—		35—	24—	20—			28—	34—		27—	35—
7. Exp. Cycle (days)	40+	31—	Amen.	Amen.	70—	21—	25—	32—	54—	38+	31—	Amen.	Amen.
8. Cycles foll. treatment (days)	20+	36—	,,	,,	38—	67—	27—	Amen.	Amen.	30+	15—	,,	,,

The animals marked with an asterisk (*) resumed the ovulatory function the following breeding season. Ovulatory cycles are indicated by means of the plus sign (+) placed after the number representing the length of the cycle; the minus sign (—) indicates non-ovulatory cycles. Amen. = amenorrhea, or cycle in excess of 100 days.

seemed possible that a higher dosage might be the answer. However, the application of 400 r to 3 animals was absolutely ineffective, though all recovered, ovulating in the next breeding season.

The series, therefore, includes one positive case. We doubt, however, that the sudden resumption of the ovulatory function in the case of No. 466 is attributable to the treatment. Two considerations favor such a conclusion: first, the absolute refractoriness of 11 other animals; and second, the fact that in the Carnegie Colony the spontaneous "recovery", with resumption of the ovulatory function, has occurred time and again. Such females often ovulate regularly for the rest of the breeding season.

If we do not feel justified in claiming a positive effect of irradiation of the hypophysis, we may conclude, on the basis of subsequent performance of the subjects, that the treatments were not in the least deleterious.

Summary. A single dose of X-ray between 60 and 400 r administered to the pituitary gland of non-ovulating rhesus monkeys failed to cause increase of ovarian size in 11 cases. The single female which ovulated might have done so without treatment as often happens in similar cases. There were no harmful sequelæ of the treatment, for in one-third of the cases spontaneous ovulation and conception occurred in the following breeding season.

10194 P

Infundibular Lesion and Pars Intermedia Activity in the Tadpole.

WILLIAM ETKIN AND LESLIE ROSENBERG. (Introduced by G. K. Noble.)

From the College of the City of New York and the American Museum of Natural History.†

Vunder[1] reported that the transplantation of a single hypophysis into an hypophysectomized Axolotl led to an excessive development of pigmentation. He ascribed this result to traumatic stimulation in the operation and supported his view by showing that the same

* This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

† Assistance in the preparation of these materials was furnished by the personnel of Works Progress Administration Official Project No. 465-97-3-67.

[1] Vunder, P. A., *Trans. Dynamics of Develop.*, 1931, **6**, 73.

result followed pricking the gland with a needle. In connection with the development of anterior lobe function in the tadpole, one of us[2] has confirmed and extended Vunder's findings. His interpretation, however, seemed unacceptable since it was there found that transplantation of even primordia into the embryo led to the development of the pigmentation weeks later and also because the effect often persisted as long as the animal could be kept, in many cases over 4 months. In a brief report of some of this material[3] it was suggested that the pituitary is under some restraint in its normal site, from which it is released on transplantation. It was further suggested that the source of this inhibition might be the nerve tract from the hypothalamus to the pars nervosa and intermedia. This tract usually called the supra-optico-hypophyseal tract has been found by the authors in the frog.

Recently we have been able to put this theory to a test through having developed an operative approach to the tadpole's pituitary. The operation was performed in *Rana sylvatica* tadpoles by exposing the entire dorsum of the brain, cutting the olfactory and one or both optic nerves and lifting the brain out sufficiently to make possible the manipulation of the infundibulum by the anterior approach. In the experimental animals the infundibulum was partly or completely destroyed by pinching with fine forceps and where possible plucking out part of the tissue. In the controls the infundibulum was merely slightly manipulated though the rest of the operation was carried out as in the experimentals. Thirteen experimental and 13 control animals survived the operation. The animals were kept to metamorphosis 12 to 23 days after operation.

Twelve of the experimental animals showed a pronounced darkening due to excessive melanophore expansion and xantholeucophore contraction during the first 2 days after operation. This darkening persisted for various periods in different animals and during its persistence the animals became darker and darker. In 7 animals in which the effect persisted for more than 12 days the accumulation of free pigment and the increase in number and expansion of the melanophores reached such a stage as to render the animals coal black, even the tail fin becoming partly opaque. Such animals approached in the intensity of color the extreme hyperpigmentation shown by the most successful pituitary graft animals.[2] None of the controls showed any unusual darkening.

Histological examination of the tissues of control and experi-

2 Etkin, W., *J. Exp. Zool.*, 1938, **77**, 347.

3 Etkin, W., *Anat. Rec.*, 1936, **64**, 75.

mental animals prepared by Bouin's fixation and Mallory's stain showed a general though not exact correlation between the completeness of the infundibular lesions and the degree of hyperpigmentation. In the cases of extreme pigmentation the pars intermedia was found to be enormously enlarged showing hyperplasia and cellular hypertrophy, the cytoplasm of the cells showing a marked increase in volume, density and chromophilia. These changes are similar to those shown by successful grafts.

In the light of the previously adduced evidence from grafts, this experiment is interpreted as indicating that the infundibular tracts to the pars intermedia normally inhibit its growth and secretory activity in the tadpole.

10195

Lactic Acid Formation by Muscles of Scorbutic Guinea Pigs.

OTTO RAHN.

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Experiments with washed cells of *Streptococcus lactis* had shown that the rate of conversion of glucose to lactic acid is always increased by addition of peptone, or of nicotinic acid.[1] Ascorbic acid acted irregularly. It proved very efficient only with injured cells, *i. e.*, cells subjected to very long centrifugation, to heating, or to long storage at 0°C. Since the mechanism of acid formation by bacteria is similar to that by animal tissues, the effect of ascorbic acid on the acid formation by muscle of normal and scorbutic guinea pigs was investigated in the hope to find that injury caused by scurvy could be repaired by ascorbic acid.

Preparation of the muscle. Killing of the animal by a blow on the head, followed by skinning and cutting of the muscle which was thrown into solid carbon dioxide, resulted in a very high lactic acid content of the muscle although the entire operation required not more than 10 minutes.

Davenport and Davenport[2] anesthetized the animal, dissected the gastrocnemii free from surrounding tissue, leaving however, blood and nerve connections intact, and after 10 minutes of rest, froze the

[1] Rahn, O., and Hegarty, C. P., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 218.
[2] Davenport, H. A., and Davenport, H. K., *J. Biol. Chem.*, 1928, **76**, 651.

muscles with solid carbon dioxide. I am obliged to Dr. H. H. Dukes of the Cornell Veterinary College for several such dissections which yielded muscles with very low initial acid content. However, the weight of the muscle was so small, especially with scorbutic animals, that the material was not sufficient for the experiment.

Finally, the entire animal was frozen in carbon dioxide during anesthesia, and all larger muscles of the hind legs were dissected in a cold storage room. The lactic acid content of the muscles was low enough to measure the rate of acid formation.

Conditions of Acid Formation. The frozen muscles were minced with a sharp knife and weighed into test tubes in the cold storage room. For most experiments, 1.0 g was used. All test tubes were placed simultaneously in a water bath, and 1 cc of a solution containing 3% of K_2HPO_4 and 2% glucose[3] was added at once to each tube. For the tests with ascorbic acid, a sufficient amount of this substance had been added to the same solution to deliver 1 mg per cc (100 mg per 100 g muscle). The temperature of the water bath remained constant within 0.1° during each experiment. It was not the same in all experiments because with the advance of summer, the temperature of the tap water rose from week to week.

Analytical Method. The small amounts of muscle made it necessary to use the micro-method of Davenport and Davenport.[2] The

TABLE I.
Lactic Acid Formation in Guinea Pig Muscle (mg per 100 g muscle).

Gastrocnemii obtained by the method of Davenport and Davenport.								
Guinea pig	Normal		Scorbutic II		Scorbutic III		Mixture II + III	
Temp.	20.0°		22.5°		23.3°		21.4°	
Ascorbic acid	0	+	0	+	0	+	0	+
Start	135	—	71	—	—	—	96	104
10 min.	—	—	—	—	148	(18)	151	196
15 "	425	442	—	—	—	—	—	—
20 "	—	—	313	317	151	145	191	207
30 "	—	—	—	—	—	—	262	212
Hind leg muscles obtained by direct freezing.								
Guinea pig	Normal		Scorbutic IV		Scorbutic V		Avg IV + V	
Temp.	25°		25°		25°		25°	
Ascorbic acid	0	+	0	+	0	+	0	+
Start	38	—	59	—	41	—	50	—
10 min.	425	445	188	215	189	222	189	219
20 "	403	517	216	165	238	212	227	189
40 "	573	521	234	210	218	264	226	237
Grand avg	467	494	213	196	215	233	214	215

[3] Meyerhof, O., *Pflüger's Archiv.*, 1921, **188**, 121.

only deviation was an increase in the length of the adsorption tube, and the cooling of the adsorption vessel on hot summer days.

Production of Scurvy. For the feeding and care of the animals, I am much obliged to Mr. Tung Shen, of the Cornell Animal Husbandry Department. The scorbutic animals were killed when the symptoms became so severe that they might not survive another night.

Results. In both sets of experiments given in Table I, the normal muscle produced about twice as much acid as the scorbutic muscle. At 20 to 25°, the maximal amount of acid was reached within 10 to 15 minutes

The second set shows a greater fluctuation of results, because of the greater heterogeneity of the muscle material. While the first set consisted of gastrocnemii only, the second set was made up of several muscles, and contained parts of sinews, fat and connective tissue which might not have been distributed quite evenly in the small samples. The 2 scorbutic guinea pigs have nearly the same rate of acid formation, and the averages show that the differences are not significant.

The injury of the enzyme complex produced by scurvy is not repaired by simple addition of ascorbic acid to the minced muscle. Neither the normal nor the scorbutic muscle showed any significant effect of the added ascorbic acid.

Ratsimamanga[4] had observed that in living guinea pigs, increasing doses of ascorbic acid result in decreasing amounts of lactic acid in muscle and blood, but in increasing amounts of glycogen in muscle and liver. The effect of ascorbic acid on injured cells of *Streptococcus lactis* which had caused this investigation is apparently not a direct effect upon the enzyme complex, but upon the repair mechanism of the cell. It suggests a relation to the observation of Tafel and Harvey[5] that wounds of scorbutic or partly scorbutic guinea pigs heal less rapidly than those of normal animals.

Summary. The excised muscle of scorbutic guinea pigs suspended in an equal volume of phosphate buffer plus glucose, produces only half as much lactic acid as the muscle of normal animals. Addition of 100 mg ascorbic acid to 100 g of muscle had no effect upon the rate or the final amount of lactic acid produced, neither with normal nor with scorbutic muscle.

[4] Ratsimamanga, A. R., *Compt. Rend. Soc. Biol.*, 1937, **126**, 1134.

[5] Tafel, M., and Harvey, S. C., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 518.

10196 P

Enzymatic Breakdown of Glycogen in Liver Extracts.

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It has been shown in previous papers[1] that dialyzed liver extracts contain an enzyme which forms glucose-1-phosphoric acid (1-ester) from glycogen and inorganic phosphate and that this enzyme is activated by adenylic acid. When 1-ester is added to liver extract, inorganic phosphate is split off due to the presence of a phosphatase. It is shown in this paper that the combined action of these two enzymes converts glycogen to glucose, a reaction which has hitherto been ascribed exclusively to a diastatic enzyme. A typical experiment is recorded in Table I. The liver of a fasted rabbit was cooled, ground in a mortar and extracted twice with ice-cold distilled water. The extract was dialyzed for 4 hours in thin collodion sacs against running tap water of 10°; it was then centrifuged at high speed for 10 minutes and used at once. Additions were made to the extract as shown in Table I, and analyses were performed before and after incubation. The glycogen, after digestion in 30% NaOH, was precipitated from boiling alcohol, centrifuged, redissolved in water and again precipitated. The fermentable sugar was determined in $HgSO_4$-$BaCO_3$ filtrates, inorganic phosphate in trichloracetic acid filtrates. The formation of hexosemonophosphate was calculated from the amount of inorganic P which was esterified during incubation.

Table I shows that addition of phosphate to the reaction mixture increases very markedly the disappearance of glycogen and that addition of adenylic acid causes a further increase. In the latter case the rate of disappearance of glycogen corresponds to 1.4 g per 100 g liver per hour which would be sufficiently rapid to meet physiological needs of blood sugar formation in that organ. Phlorhizin, which is known to inhibit the disruptive phosphorylation of glycogen in muscle hash or extract,[2] also has an inhibitory effect in liver extracts.

By means of acid and alkaline fermentation (Somogyi[3]) it was

1 Cori, G. T., Colowick, S. P., and Cori, C. F., *J. Biol. Chem.*, 1938, **123**, 375; 1938, **124**, 543.

2 Lundsgaard, E., *Biochem. Z.*, 1933, **264**, 209; Ostern, P., Guthke, J. A., and Terszakowec, *Z. physiol. Chem.*, 1936, **243**, 9.

3 Somogyi, M., *J. Biol. Chem.*, 1937, **119**, 741.

TABLE I.

Enzymatic Breakdown of Glycogen in Dialyzed Liver Extract.

10 cc of reaction mixture corresponds to 5 g of liver. Incubation period 1 hr, temperature 37°.

Composition of reaction mixture	Changes during incubation (mg per 10 cc mixture)			
	Glycogen	Hexosemono-phosphate (as hexose)	Fermentable sugar	% glycogen accounted for
1. 1% glycogen .004 M $MgSO_4$	—12		+ 6	50
2. 1% glycogen .004 M $MgSO_4$ .02 M phosphate pH 7.2	—39	(+0.3)	+30	77
3. 1% glycogen .004 M $MgSO_4$ .02 M phosphate .001 M adenylic a.	—70	+19	+48	96
4. Same as 3. .005 M phlorhizin	—40			

ascertained that the fermentable sugar formed was mostly glucose. The glucose formation was highest in the experiment with adenylic acid in which case there was also an accumulation of hexosemonophosphate, its rate of formation being apparently greater than its rate of breakdown to glucose and inorganic phosphate. It may be mentioned that in muscle extract such a breakdown of hexosemonophosphate does not occur, because phosphatases are absent.

The effects of addition of phosphate, adenylic acid and phlorhizin are characteristic for the phosphorylating enzyme system. It is difficult to prepare an active diastase from liver and when the organ is first washed free of blood, hardly any diastase can be extracted (Davenport[4]). Furthermore, the diastatic enzyme causes a stepwise breakdown of glycogen, so that after short periods of incubation, such as were used in the experiment in Table I, dextrines predominate. Only after much longer periods of incubation do larger amounts of maltose and glucose make their appearance in the reaction mixture.[5]

It is concluded that there is present in dialyzed liver extracts an enzyme system which after addition of phosphate buffer and adenylic acid is capable of rapid glucose formation from glycogen. It seems possible that the blood sugar of mammals is produced by this enzyme system rather than by a diastase. In this connection it is suggestive that when liver slices are shaken in oxygenated phosphate-Ringer's solution, addition of phlorhizin exerts an inhibitory effect on the breakdown of glycogen.

4 Davenport, H. A., *J. Biol. Chem.*, 1926, **70**, 625.

5 Somogyi, M., *J. Biol. Chem.*, 1938, **124**, 179.

10197

Therapeutic Effect of 4,4′-Diamino-Diphenyl-Sulfone, Corresponding Sulfide and Acetyl Derivatives in Streptococcic Infection.

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Although the therapeutic effect of sulfanilamide (para-amino-benzene-sulfonamide) in experimental beta-hemolytic streptococcus infection of mice has been definitely established, Buttle, Stephenson and others[1] have discovered that 4,4′-diamino-diphenyl sulfone,* (previously described in the chemical literature by Fromm and Wittman[2]) has still greater therapeutic effect upon the same infection. Buttle, *et al.*, stated that the sulfone compound is tolerated by mice in 5 mg doses given by mouth. Two mg can be given daily to normal mice. Although the product is more toxic for mice than sulfanilamide, the authors mentioned that it is not more toxic for normal rabbits and monkeys. They found that single doses of 2 g per kilo of body weight are tolerated by rabbits, and maintain that doses of 0.4 mg of diamino-diphenyl-sulfone are as effective as 40 mg of sulfanilamide.

Fourneau, Trefouël, etc.[3] found that sulfanilamide is tolerated by mouth in a dose of 2.5 g per kilo of body weight (or 50 mg per 20 g of mouse). The maximum tolerated doses per kilo of body weight for other compounds tested are as follows: diamino-diphenyl-sulfide, 0.5 g; diamino-diphenyl-sulfone, 0.1 g; diacetyl-amino-diphenyl-sulfide, more than 10 g; and diacetyl-amino-diphenyl-sulfone, 10 g. The authors found the minimum therapeutic dose in streptococcic infection of mice to be 0.001 g (per 20 g of mouse) for diamino-diphenyl-sulfide; 0.00005 g diamino-diphenyl-sulfone; 0.001 g, diacetyl-amino-diphenyl-sulfide; 0.00005 g, diacetyl-amino-diphenyl-sulfone; and 0.001 g for sulfanilamide. They state, however, that it is difficult to determine the minimum therapeutic dose,

* All of these compounds are 4,4′- but throughout the paper we will refer to them as diamino-diphenyl-sulfone, diamino-diphenyl-sulfide, etc.

1 Buttle, G. A. H., Stephenson, D., Smith, S., Dewing, T., and Foster, G. E., *Lancet*, June 5, 1937, p. 1331.

2 Fromm, E., and Wittmann, J., *Ber. dtsch. chem. Ges.*, 1908, **41**, 2269.

3 Fourneau, E., Trefouël, J., and Mme. J., Nitti, F., and Bovet, D., *Bull. l'Acad. Med.*, 1937, **118**, 210.

and, in general, they consider as the "active dose" the one which gives the same results as obtained under the same conditions with 0.0025 g of sulfanilamide given 2 days in succession by mouth.

Bauer and Rosenthal[4] found diamino-diphenyl-sulfone to be 30 times more active than sulfanilamide. They also found the maximum tolerated dose of diamino-diphenyl-sulfone to be 0.25 g per kilo of body weight given orally to mice.

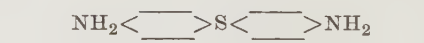
Cooper, Gross, and Lewis[5] found that diacetyl-amino-diphenyl-sulfone is more efficacious than sulfanilamide in the treatment of mice infected with beta-hemolytic streptococcus strain C 203.

Feinstone, Ott, Bliss and Long[6] found diamino-diphenyl-sulfone to be more active in experimental streptococcal infections than sulfanilamide and highly toxic for mice.

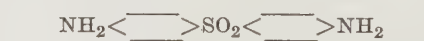
In view of the reported high therapeutic efficiency of diamino-diphenyl-sulfone, we found it of interest to determine whether this product is superior in its therapeutic effect to sulfanilamide. We were particularly interested in comparing the two products and ascertaining how much more effective the sulfone is as compared with sulfanilamide.

At the same time the diamino-diphenyl-sulfide and the acetyl derivatives were drawn into our study. The toxicity of these compounds also was studied.

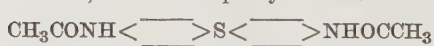
Chemistry. The structural formulæ of these products are presented as follows:



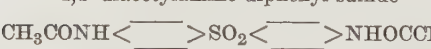
4,4'-diamino-diphenyl-sulfide



4,4'-diamino-diphenyl-sulfone



4,4'-diacetylamino-diphenyl-sulfide



4,4'-diacetylamino-diphenyl-sulfone

The above products are crystalline solid compounds which are only sparingly soluble in water at room temperature and slightly

4 Bauer, H., and Rosenthal, S. M., *Public Health Rep.*, 1938, **53**, 40.

5 Cooper, F. B., Gross, P., and Lewis, M., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 375.

6 Feinstone, W. H., Bliss, E. A., Ott, E., and Long, P. H., *Bull. Johns Hopkins Hosp.*, 1938, **62**, 565.

more soluble in hot water. They are more soluble in alcohol or acetone.

The diamino-diphenyl sulfone was made from pure diamino-diphenyl-sulfide by oxidation and all products were recrystallized several times in order to obtain them in a condition of the highest purity.

The toxicity was studied on rabbits by oral administration. For this purpose a small rubber tube was introduced as far as possible into the oesophagus of the rabbit, and by means of a syringe the drug, suspended in aqueous solution was forced through the tube into the stomach of the animal. The toxicity of sulfanilamide was reported by us previously.[7, 8]

TABLE I.
Toxicity Tests in Rabbits *Per Os.*

Drug	Dose per kilo of body wt.	
	Tolerated g	Lethal g
No. 2341	0.3	
4,4'-diamino-diphenyl-sulfide		0.5
		0.5
		0.75
No. 2344	0.3	
4,4'-diamino-diphenyl-sulfone	0.3	
	0.5	
	0.5	
		0.75
No. 2345	2.0	
4,4'-diacetyl-amino-diphenyl-sulfide	3.0	
	5.0	
		6.0
		8.0
No. 2356	10.0	
4,4'-diacetyl-amino-diphenyl-sulfone	15.0	
	15.0	
	20.0	
Sulfanilamide	1.5	2.0

From the data presented in Table I one can see that diamino-diphenyl-sulfone has been tolerated in a dose of 500 mg per kilo of body weight, while the tolerated dose of sulfanilamide is 1.5 g. Diamino-diphenyl-sulfone is, therefore, about 3 times more toxic for the rabbit. The diamino-diphenyl-sulfide is 5 times more toxic

7 Raiziss, G. W., Severac, M., and Moetsch, J. C., *J. Chemoth.*, 1937, **14**, 1.

8 Raiziss, G. W., Severac, M., Moetsch, J. C., and Clemence, L. W., *J. Chemoth.*, 1938, **14**, 91.

than sulfanilamide. On the other hand, the corresponding diacetyl derivative of the sulfone is tolerated in very high doses, up to 20 g per kilo of body weight. This is undoubtedly due to the fact that the absorption of the product by the intestinal route is very slow and incomplete. In fact, when very large doses of this drug were given to animals, we observed a more frequent excretion of feces which contained considerable quantities of the drug. The diacetylamino-diphenyl-sulfone could easily be isolated from the excreta by extracting the drug with acetone. Upon recrystallizing, a pure product was isolated. Our conclusion, however, is that the diacetyl compounds are considerably less toxic than the diamino products.

Therapeutic Effect in Experimental Streptococcic Infection of Mice. In this study mice were infected with a virulent culture of beta-hemolytic streptococci of the strain C 203, 0.5 cc of which in these experiments killed mice in a dilution of 10^{-6} (1,000,000) to 10^{-7} (10,000,000). Mice were infected with about 1000 minimum lethal doses of the culture by intraperitoneal injection. An hour and a half later, treatment was given by mouth. These treatments were continued daily for 4 days more. The mice were kept under observation for 28 days.

In Tables II, III, IV, and V, a summary of various individual experiments is presented. Table II presents results of treatment by ascending doses of diamino-diphenyl-sulfone, 0.0001 g causing only a 40% survival of mice, an indication that the dose was not effective; 0.0005 g showed a good therapeutic result, particularly in

TABLE II.

Percentage of Mice Surviving Effects of Infection Following Administration of 4,4'-diamino-diphenyl-sulfone.

Daily dose, g	No. of mice used	7 days %	14 days %	21 days %	28 days %
0.0001	10	60	50	40	40
0.0005	30	90	80	70	66⅔
0.001	15	80	66⅔	66⅔	66⅔
0.002	5	100	80	80	80

TABLE III.

Percentage of Mice Surviving Effects of Infection Following Administration of 4,4'-diamino-diphenyl-sulfide.

Daily dose, g	No. of mice used	7 days %	14 days %	21 days %	28 days %
0.0005	10	40	30	30	30
0.001	15	53	33⅓	33⅓	33⅓
0.002	5	20	20	20	20
0.003	10	50	50	40	40
0.005	5	20	20	20	20

TABLE IV.

Percentage of Mice Surviving Effects of Infection Following Administration of 4,4′-diacetyl-amino-diphenyl-sulfone.

Daily dose, g	No. of mice used	7 days %	14 days %	21 days %	28 days %
0.0001	10	70	40	30	20
0.0005	10	60	20	20	20
0.001	20	85	75	70	55
0.002	30	77	60	60	60
0.005	5	80	80	80	80

TABLE V.

Percentage of Mice Surviving Effects of Infection Following Administration of 4,4′-diacetyl-amino-diphenyl-sulfide.

Daily dose, g	No. of mice used	7 days %	14 days %	21 days %	28 days %
0.0005	5	20	20	20	20
0.001	5	0	0	0	0
0.002	25	84	68	56	56
0.005	15	73	60	53	53
0.010	10	80	80	50	50

the first 14 days. The administration of 0.001 g did not materially improve the results. 0.002 g, however, gave the best effect. Comparing the survival of mice following the sulfone with that following sulfanilamide, one may designate 0.0005 g to be the minimum therapeutic dose. Approximately the same result is obtained with

TABLE VI.

Percentage of Mice Surviving Effects of Infection Following the Administration of Sulfanilamide, 4,4′-diamino-diphenyl-sulfone and Allied Compounds.

Drug	Daily dose, g	No. of mice used	7 days %	14 days %	21 days %	28 days %
Sulfanilamide	0.002	85	67	52	51	37
	0.005	25	68	48	48	48
	0.010	40	75	68	65	60
No. 2341 4,4′-diamino-diphenyl-sulfide	0.003	10	50	50	40	40
No. 2344 4,4′-diamino-diphenyl-sulfone	0.0005	30	90	80	70	66⅔
	0.001	15	80	66⅔	66⅔	66⅔
	0.002	5	100	80	80	80
No. 2345 4,4′-diacetyl-amino-diphenyl-sulfide	0.002	25	84	68	56	56
	0.005	15	73	60	53	53
No. 2356 4,4′-diacetyl-amino-diphenyl-sulfone	0.002	30	77	60	60	60
	0.005	5	80	80	80	80

0.005 g of sulfanilamide. Contrasting the minimum therapeutic effect of diamino-diphenyl-sulfone with that of sulfanilamide we may conclude that the sulfone is about 10 times as therapeutic as sulfanilamide.

In Table VI we present a summary of results obtained with all 4 compounds used in these studies. The difficulties are obvious in selecting a dose which is considered to be the minimum therapeutic. It requires a careful study of individual experiments and of the averages. With certain reservations we consider the minimum therapeutic doses given daily for 5 consecutive days, according to the type of experiments performed, to be: for sulfanilamide, 0.005 g, for 4,4′-diamino-diphenyl-sulfone, 0.0005 g, for diamino-diphenyl-sulfide, 0.003 g, for di(acetyl-amino)-diphenyl-sulfone, 0.001 g, and for di(acetyl-amino)-diphenyl-sulfide, 0.002 g.

Conclusions. 1. 4,4′-diamino-diphenyl-sulfone is about 3 times as toxic for the rabbit as is sulfanilamide; 4,4′-diamino-diphenyl-sulfide is 5 times as toxic. 2. The corresponding diacetyl derivatives of sulfone and sulfide are less toxic than sulfanilamide. 3. The minimum therapeutic doses given daily for 5 consecutive days are as follows: for sulfanilamide, 0.005 g, for 4,4′-diamino-diphenyl-sulfone, 0.0005 g, for diamino-diphenyl-sulfide, 0.003 g, for di(acetyl-amino)-diphenyl-sulfone, 0.001 g, and for di(acetyl-amino)-diphenyl-sulfide, 0.002 g. 4. In mice infected with beta-*Streptococcus hemolyticus,* 4,4′-diamino-diphenyl-sulfone is therapeutically active in a dose 10 times smaller than that required by sulfanilamide. This indicates a considerably higher therapeutic efficacy of the sulfone over sulfanilamide.

10198

Occurrence of Positive Vaginal Smears in Spayed Mice.*

ISABELLA H. PERRY.

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We[1] reported that spayed mice painted with 1:2:5:6 dibenzanthracene had positive vaginal smears sporadically. In preparing to

* Aided by grants from the Christine Breon Fund for Medical Research and the International Cancer Research Foundation.

1 Perry, I. H., and Ginzton, L., *Am. J. Ca.*, 1937, **29**, 680.

TABLE I.

Vaginal Smears in Spayed Mice.

	16 II	17 II	18 II	19 II	20 II	22 III	5 VI	6 VI	7 VI	8 VI	9 VI	†10 VI	11 VI	12 VI	24 VI	25 VI	26 VI	27 VI	28 VI	29 VI	30 VI	1 VII	2 VII	15 VII
Treatment wks	11					16	28															32		
Age wks	27						44															48		
1	—	∓	±	—	—	—	+	+	+	—	±	—	—	—	+	+	±		+	+	+	+	∓	—
2	±	+	+	+	∓	—	*																	
3	—	+	∓	—	+	—	+	+	+	+	+	±	+	—	—	—	—		+	±	+	±	—	—
4	—	+	∓	—	—	—	+	—	—	—	—	—	—	—	±	±	—		+	+	+	+	∓	—
5	—	+	+	∓	—	—	+	—	—	+	—	—	—	—	*									
6	±	+	+	+	—	—	∓	—	—	—	—	—	—	—	+	±	*							
7	—	+	+	—	±	—	+	—	—	—	—	—	—	—	+	—	—		+	+	+	+	∓	
8	±	+	∓	±	±	*																		
9	—	+	∓	∓	—	+	—	—	—	—	—	—	—	—	±	—	—		+	±	+	±	∓	—
10	—	+	+	+	+	—	+	+	—	—	—	—	—	+	+	—	—		+	+	+	+	∓	—
11	—	+	∓	—	—	—	+	—	—	—	—	—	—	—	+	+	+		+	+	+	±	∓	—
12	—	+	—	—	—	—	*																	
13	±	+	∓	∓	—	—	*																	
14	∓	∓	+	—	—	—	*																	
15	±	+	∓	—	—	—	*																	
C1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—		+	+	+	+	+	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—		+	+	+	+	+	—
3															—	—			—	—	—	—	—	+
4 Separately caged															—	—			—	—	—	—	—	—
5															—	—			—	—	—	—	—	—
6															—	—			—	—	—	—	—	—

*Dead. †All have papillomas treatment stopped.

repeat this experiment we[2] found that effective amounts of estrone may be transmitted from animal to animal. The animals used in this experiment were kept segregated as a group throughout the experiment. They were spayed. No accessory ovaries were found at autopsy. Smears were made twice daily for 5 days before treatment was begun; and they were all negative. Smears were omitted until 11 weeks after treatment began, to avoid pseudo-estrus[3] effects from mechanical stimulation of the vagina. Smears were made twice a week throughout the rest of the experiment, which terminated 41 weeks after treatment with 1:2:5:6 dibenzanthracene was begun. If a positive or plus-minus smear was found all animals were smeared daily until there was a return to negative. Two untreated spayed controls were kept in the box with the treated mice. Later 4 old spayed females with uterine implants of dibenzanthracene were segregated and kept as additional controls. They were caged separately. Table I shows the positive vaginal smears. Periods when all were negative or plus-minus are omitted for brevity. Estrus was occasionally observed in the treated animals and in the controls.

Frank[4] has reported positive vaginal smears in human post-menopausal and castrate cases. Loeb[5] has reported hyperplasia of the epithelium of the cervix and vagina of old mice. These data suggest that occasional definitely positive vaginal smears develop in old or sexually involuted females. Whether the response is to local or general stimuli is unknown, as is its significance.

10199

Estrogenic Activity of 3-4 Benzpyrene.*

ISABELLA H. PERRY

From the Division of Pathology, University of California Medical School, San Francisco.

Cook and Dodds[1] reported that 3 of the carcinogenic hydrocarbons were estrogenic. The carcinogenic and estrogenic effects were

[2] Perry, I. H., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 122.

[3] Emery, F., and Schwabe, E. L., *Anat. Rec.*, 1936, **64**, 147.

[4] Salmon, U. J., and Frank, T., PROC. SOC. EXP. BIOL. AND MED., 1936, **33**, 621.

[5] Burns, E. L., Suntzeff, V., Muskup, M., and Loeb, L., *Am. J. Ca.*, 1938, **32**, 534.

* Aided by grants from the Christine Breon Fund for Medical Research and the International Cancer Research Foundation.

[1] Cook, J. W., and Dodds, E. C., *Nature*, 1933, **131**, 205.

not quantitatively or temporally related. A causal relationship is a matter for speculation. Of these 3 estrogenic carcinogens 3-4 benzpyrene has been the most extensively experimented with. Cook and Dodds injected 100 mg of the carcinogenic hydrocarbon in oil subcutaneously into each of 10 rats. Three of the 10 rats receiving 3-4 benzpyrene had positive vaginal smears for 150 hours.

Carminati[2] reported that 3-4 benzpyrene was not estrogenic. He used the same dose as Cook and Dodds for 2 rats, and various smaller doses for 9 other rats.

We injected 31 mg of 3-4 benzpyrene in 0.75 cc of lard subcutaneously into each of 4 mice standardized according to Palmer's[3] method. In 48 hours 3 had positive vaginal smears. On the fifth day the fourth mouse had plus-minus reaction. On the sixth day all were negative, and remained so. Twenty spayed but not standardized mice were injected subcutaneously with 4 mg of 3-4 benzpyrene in lard. They did not develop estrus. Much of the injection mass was present in the 2 mice dying without tumor and a moderate amount present in those developing tumors so the estrogenic amount must lie between 31 and 4 mg.

One of the 4 animals receiving 31 mg of 3-4 benzpyrene died in the third week. A second animal died at 2½ months. Neither of these had tumors. One month after being injected 2 animals developed tumors at the site of injection. One of these animals was the one that did not go into estrus. The other was not in estrus when the tumor began. The animals were killed at 3½ and 4½ months when the tumors measured about 4x3x3 cm. The tumors were sarcomas with considerable cytological variation. There were no metastases. Five to 10 mg of benzpyrene subcutaneously produces tumors in about 3½ months.[4] The development and duration of the tumors in the massively injected mice does not appear to vary significantly from the response to smaller doses.

Three controls injected with 1 cc of lard did not go into estrus or develop tumors.

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2 Carminati, V., *Tumori*, 1935, **9**, 106.

3 Palmer, Allan, PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 123.

4 Fieser, L. F., Fieser, Mary, Hershberg, E. B., Newman, M. S., Seligman, A. M., and Shear, M. J., *Am. J. Cancer*, 1937, **29**, 260.

10200

A Proposed Mouse Protection Unit for Anti-Meningococcus Serum.

Sara E. Branham, Margaret Pittman, Geoffrey Rake and Henry W. Scherp.

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The use of mucin for the enhancement of the infectivity of meningococci in the mouse has now been studied for over 5 years.[1] The first use of this technic in the titration of the protective action of sera was reported over 3 years ago.[2] Subsequently, other laboratories have reported the titration of protective antibodies in antimeningococcus serum and in each case the details of technic employed have varied slightly or markedly.[3-9] In order that values obtained in the titration of antimeningococcus sera in various laboratories may be comparable, that figures obtained by the use of the test may have a wide application, and that sera used for therapeusis may be studied on a basis which allows quantitative comparison, it has seemed advisable to set up and distribute a tentative standard polyvalent serum as a control and to assign to this serum certain values so that other sera may be referred to it quantitatively. It has also seemed desirable that a uniform technic for performing the mouse protection test be used.

In order to obtain a polyvalent serum and data for this purpose, a serum (of which ample amounts were available) was selected because it resembled closely the older control serum M 18 in its protective capacity and other immunological reactions. This has been designated M 19. Repeated estimations of antibody nitrogen for Group I* were carried out with this serum and it was determined that the

1 Miller, C. P., *Science*, 1933, **78**, 340.

2 Rake, G., *J. Exp. Med.*, 1935, **61**, 545.

3 Miller, C. P., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 1140.

4 Rake, G., *Ibid.*, 1935, **32**, 1175.

5 Cohen, S., *J. Immunol.*, 1936, **30**, 203.

6 Mishulow, L., Melman, M., and Sklarsky, R., *J. Lab. and Clin. Med.*, 1936, **21**, 406.

7 Miller, C. P., and Castles, R., *J. Inf. Dis.*, 1936, **58**, 263.

8 Rake, G., *Can. Pub. Health J.*, 1937, **28**, 265.

9 Pittman, M., Branham, S. E., and Sockrider, E. M., *U. S. Pub. Health Rep.*, 1938, **53**, 1400.

* By Group I is meant only those strains known as Type I and Type III; by Group II only those strains known as Type II.

serum contained 0.65 mg of antispecific polysaccharide nitrogen[10] per cubic centimeter. It has been shown[8] that the Group I protective power of antimeningococcus serum probably parallels the type-specific antibody nitrogen content. This has also been found to be the case with antipneumococcus sera. In the latter case 1.00 mg of antibody nitrogen is found to be equivalent to approximately 1000 protective units, a convenient figure. In view of these facts, it seemed logical to give a tentative value of 650 protective units per cubic centimeter for the Group I titer of serum M 19, and this figure has been tentatively adopted.

The present lack of sufficiently pure Group II specific substance has precluded the use of antibody nitrogen estimation in arriving at a figure for the Group II protective antibodies. In common with all other polyvalent antimeningococcus sera so far investigated, the Group II titer for M 19 is far lower than the Group I. On the basis of the protective capacity of the serum against Group II strains as compared to the like capacity against Group I, a figure of 25 protective units per cc would seem to be approximately correct and it is suggested that this figure be tentatively adopted for comparative purposes. This tentative protective unit for both Groups I and II is for purposes of research, and is not to be considered as an official standard unit for antimeningococcus serum.

For the mouse protection test the cultures used, whether of Group I or Group II, should be of maximum virulence. This means that 1 cc of a 10^{-9} dilution which contains on the average 2 organisms should kill over 50% of mice on intraperitoneal inoculation. Such strains are available. The virulence of these strains can usually be maintained by the preparation and use of cultures which have been dried from the frozen state or by weekly passage on serum dextrose agar. It can be more certainly maintained by passage through mice at least once a week and maintenance on serum dextrose agar or by daily passage on 4-5% blood agar.

In order that this high virulence may be rendered apparent and usable it is, of course, essential that mucin be used. Granular mucin (Wilson) is recommended, and suspensions are made according to the description of Miller and Castles.[7] †

To obtain consistent results with small numbers of mice it is advisable that a pure line inbred stock of proven susceptibility be used

10 Scherp, H. W., and Rake, G., *J. Exp. Med.*, 1936, **63**, 547.

† In the past different lots of the mucin preparation have varied considerably and this has introduced an element of difficulty. Recently, the Wilson Laboratories have supplied us with a new preparation which gives most satisfactory results and which, it is believed, can be reproduced at will.

at weights between 16 and 20 g. Such a stock is available in this country as the so-called "Swiss" mouse.

In carrying out the actual test, blood agar cultures 4 to 6 hours old are used. The culture is suspended in broth and standardized to give 2,000,000,000 organisms per cc by some method such as the Gates Turbidimeter or comparison with standard suspensions of silica. Dilutions from this suspension are made at 10^{-1} and 10^{-2} in broth and at 10^{-3} through 10^{-9} in mucin.

In the meanwhile, progressive twofold dilutions of the control, or standard, serum and of the unknown sera have been made in saline. Three dilutions are used, as a rule, but more may be employed as long as both control and unknown have the same number. The dilutions most suitable for the control serum will be known beforehand; those for the unknown can be determined roughly in preliminary tests. A minimum of 5 mice are inoculated with each of the 3 (or more) dilutions, which are so prepared that the required dose of serum will be contained in 0.5 cc. Serum inoculations are given intraperitoneally. An hour later, all mice receiving serum are given 1 cc of a 10^{-3} or 10^{-4} dilution of meningococci in mucin intraperitoneally. Luer-Lok syringes and 24 gauge needles are suggested for this purpose. A control of the culture virulence is carried out in every test by inoculating a minimum of 3 mice in each dilution with 10^{-8} and 10^{-9} suspensions of meningococci in mucin (20 and 2 organisms approximately).

The mice are observed for 96 hours after the inoculation and deaths recorded in the usual manner. Given a control serum of agreed quantitative value in protective units the titers of unknown sera are worked out by the use of the formulæ suggested by White[11] or by Reed and Muench.[12]

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[11] White, B., *The Biology of the Pneumococcus*, 1938, N. Y.

[12] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

10201

Chemical Composition of Transverse Metaphyseal Bands Produced in Growing Bones by Phosphorus Ingestion.

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Transverse bands of increased density in the metaphyseal region of growing long bones, caused by the ingestion of elemental phosphorus, were first described by Wegner.[1] The exact chemical nature of these bands has not been known.

An increase in the absolute and relative amount of inorganic phosphorus in the metaphyseal regions has been suggested. Parks, Goodwin, and Kajdi[2] have shown that lead lines, seen in the X-rays of growing long bones in children suffering from lead poisoning, are produced by the actual deposition of lead in the newly formed bones. Bone growth is most active in the metaphyseal region and the lead deposited there may be demonstrated roentgenologically as a transverse band of increased density. Caffey[3] found that these zones of increased density contained 0.7 g of lead in each gram of bone analyzed. There was only 0.19 g of lead in each gram of bone taken from the shaft of the same bones. In a previous report I have shown that phosphorus causes increased density only in the metaphyses and not in callus of healing fractures.[4]

Harris[5] and others have contended that the phosphorus lines represent zones of arrested longitudinal growth. In a previous report,[4] I postulated the theory that the toxicity of ingested phosphorus causes retardation of the reproduction of new cartilage cells in the epiphyseal plate, while mineral salts continue to be deposited at approximately the normal rate.

The theory that ingested elemental phosphorus causes a marked stimulation of osteoblastic activity in the metaphyseal regions has also been suggested.[2]

If the phosphorus bands were produced by an increase in the percentage or the ratio of phosphorus in this dense bone, a marked

* The author wishes to acknowledge the assistance given by W. J. Highman, Jr., who made the chemical analyses of the specimens.

1 Wegner, G., *Virchows Arch. f. path. Anat.*, 1872, **55**, 11.

2 Parks, E. A., Jackson, D., Goodwin, T. C., and Kajdi, L., *J. Ped.*, 1933, **3**, 265.

3 Caffey, John, *Radiol.*, 1931, **17**, 957.

4 Adams, C. O., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 449.

5 Harris, H. A., *Brit. J. Radiol.*, 1931, **4**, 561, 622.

change from the normal chemical content should be detected by quantitative analyses of the calcium and phosphorus elements.

Twenty-nine young rabbits from 5 litters were used. To each of half of the animals of each litter was given, in addition to the stock diet, a pill of 0.6 mg of yellow phosphorus each day. Phosphorus lines were demonstrated roentgenologically during the second week. Three of the animals which showed wide, dense zones of transverse density in the metaphyseal regions and 3 normal litter mates were selected for this study (Fig. 1).

FIG. 1. FIG. 2.

FIG. 1.

Roentgenogram of the tibia and fibula of a young rabbit which was sacrificed after being fed 0.6 mg of elemental phosphorus each day for 9 days. The transverse bands of increased density are distinctly defined.

FIG. 2.

Litter mate control. The diet was identical, but no phosphorus was added.

The right tibiæ were split longitudinally and the metaphyseal region dissected out. In the specimens from the animals which had been receiving phosphorus, the transverse zone of increased density in the metaphysis was grossly visible, and readily excised. The corresponding region of the bone was dissected from the control animals. A portion of the cortex from the shaft of these bones was taken as a further control.

The 12 samples were dried at 80°C and extracted with ether in a Soxhlet apparatus. The fat-free bone was then weighed, ashed in a muffle furnace at 500°C and reweighed. The ash was dissolved in dilute HCl and made up to 25 cc total volume with distilled water.

TABLE I.
Results of Chemical Analysis of the Dry Fat-Free Bones.

Day of Experiment	Calcium				Phosphate				Ratio Ca/P			
	Cortex		Metaphyseal Region		Cortex		Metaphyseal Region		Cortex		Metaphyseal Region	
	No P %	With P %	No P %	With P %	No P %	With P %	No P %	With P %	No P	With P	No P	With P
9	25.6	23.2	21.7	22.2	13.3	12.5	10.5	11.3	1.93	1.86	2.07	1.96
21	25.1	22.3	18.6	21.2	12.3	12.0	9.1	10.3	2.04	1.86	2.04	2.06
26	24.8	24.3	18.1	23.5	12.6	11.7	9.0	11.5	1.97	2.08	2.01	2.04

Determinations of the calcium and phosphorus of these samples were made in duplicate.[6, 7]

The percentage of calcium and the percentage of phosphorus were essentially the same in all specimens. The ratio of the phosphorus and calcium was even more constant. Any variation shown in these ratios is entirely within the range of analytical error (Table I).

These results indicate that the phosphate-calcium ratio in the bones is not changed by the ingestion of phosphorus.

Conclusions. 1. The bone in the phosphorus bands produced in the metaphyses of growing long bones by the ingestion of phosphorus is of normal chemical composition. 2. The increased density represents deposition of radio-opaque salts in greater amount per unit of tissue calcified, since calcification and ossification have continued unchecked while the cartilaginous growth was inhibited. 3. This observation constitutes additional evidence that the zones of increased density in metaphyses of growing bones, produced by ingested phosphorus, are in fact "growth arrest lines."

10202

Heptaldehyde as a Tumor Inhibitor.*

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In connection with studies on tumor-inhibition by oil of wintergreen, Strong identified heptaldehyde in the active fraction of the oil.[1, 2, 3] He then fed a diet containing commercial heptaldehyde to mice with spontaneous tumors, and observed liquefaction in most of the tumors followed in many cases by regression. More recently, Strong and Whitney reported a similar response in dogs with spon-

[6] Fiske, C. H., and Logan, M. A., *J. Biol. Chem.*, 1931, **93**, 211.

[7] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **46**, 285.

* Supported by the Jonathan Bowman Cancer Fund and the Wisconsin Alumni Research Foundation.

[1] Strong, L. C., *Am. J. Ca.*, 1936, **28**, 550.

[2] Strong, L. C., *Am. J. Ca.*, 1938, **32**, 227.

[3] Strong, L. C., *Science*, 1938, **87**, 144.

taneous mammary tumors when heptaldehyde was injected subcutaneously.[4]

The question arose whether heptaldehyde would inhibit all tumors or whether its action was restricted to certain tumor types. We have, therefore, studied the effect of heptaldehyde on 5 kinds of mouse tumors: (1) spontaneous mammary adenocarcinoma in strain A mice, (2) primary ear tumor induced by ultraviolet light, (3) primary epithelial tumor induced by painting benzpyrene, (4) primary sarcoma induced by the subcutaneous injection of benzpyrene, (5) transplantable spindle-cell sarcoma originally induced by benzpyrene.

The heptaldehyde (Eastman technical) was fed mixed in various amounts with Steenbock stock ration† (Table I). In general the mice were placed on the diets when the tumors were approximately one centimeter in diameter, although some of the spontaneous tumors were larger. The mice with transplantable tumors were given the various diets from the day of inoculation. In each series the animals were divided such that the tumors in the various groups were of comparable sizes. The tumors were measured at weekly intervals and all mice were carefully autopsied.

When 2% heptaldehyde was fed to mice bearing U.V. tumors, the tumors grew more slowly than in animals on the stock diet. However, these mice ate so little that the limiting factor in the

TABLE I.
Effect of Heptaldehyde on Survival of Tumor Mice.

Type of Tumor	Amt. of Heptaldehyde %	No. of Mice	Survival Avg No. days
Ultraviolet irradiation	0.	30	39.7
" "	0.4	10	18.8
" "	0.8	20	31.5
" "	2.0	10	19.4
Benzpyrene injected	0.	3	33.0
" "	0.8	4	27.0
Benzpyrene painted	0.	12	16.6*
" "	0.8	13	43.8
Benzpyrene transplant	0.	36	24.3
" "	0.8	6	12.0
" "	2.0	23	25.0
Spontaneous mammary	0.8	11	17.7

*The cause of early death of the controls in this group is unknown. In a repetition of this experiment, the survival of mice on both diets was the same.

[4] Strong, L. C., and Whitney, L. F., *Science*, 1938, **88**, 112.

† In one series the heptaldehyde was also injected subcutaneously, but the resulting ulceration and the high mortality of the injected animals rendered the results meaningless.

growth of the tumors appeared to be the reduced caloric intake rather than the heptaldehyde. This was demonstrated by feeding stock ration to tumor-bearing mice in the small amounts consumed by the animals receiving 2% heptaldehyde. Under these conditions the growth rate of the tumors was also markedly restricted.

When 0.8% heptaldehyde was fed, food consumption was more nearly normal. This level approximates the amount fed by Strong. However, in our experimental animals the tumors grew at the same rate as those in the controls. The feeding of heptaldehyde failed to prolong the life of the tumor-bearing animals; in fact in several groups it appeared to hasten death (Table I). Nor did it alter the character of the tumors. Of 97 tumor-bearing mice treated, only one small spontaneous tumor regressed and the animal in question died shortly thereafter. Heptaldehyde also failed to inhibit the growth of the Flexner-Jobling rat carcinoma. The discrepancy between our results and those of Strong demonstrate that heptaldehyde is not a universal tumor inhibitor.

10203 P

Production of Cirrhosis in Fatty Livers with Alcohol.*

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It was previously shown that depancreatized dogs develop cirrhosis of the liver when maintained with insulin and a diet containing meat, sucrose, bone ash and vitamins. The cirrhosis was observed as early as 2.6 years after pancreatectomy and was preceded by the infiltration of massive amounts of fat, the latter appearing as a rule within 20 weeks after excision of the gland. It was concluded that the scar tissue developed in response to the presence of the large amounts of fat in the liver. In the present study cirrhosis in normal dogs is shown to occur when the feeding of large amounts of alcohol is superimposed upon a previously established fatty liver.

For 30-35 days each dog received daily 10 g lard and 7 g lean meat per kilo. Vitamin sources and Cowgill's salt mixture were fed throughout the experiment. At the end of this period, the adminis-

* Aided by grants from the Christine Breon Fund for Medical Research, and by W.P.A. (O.P. 465-03-3-631, Unit A 6).

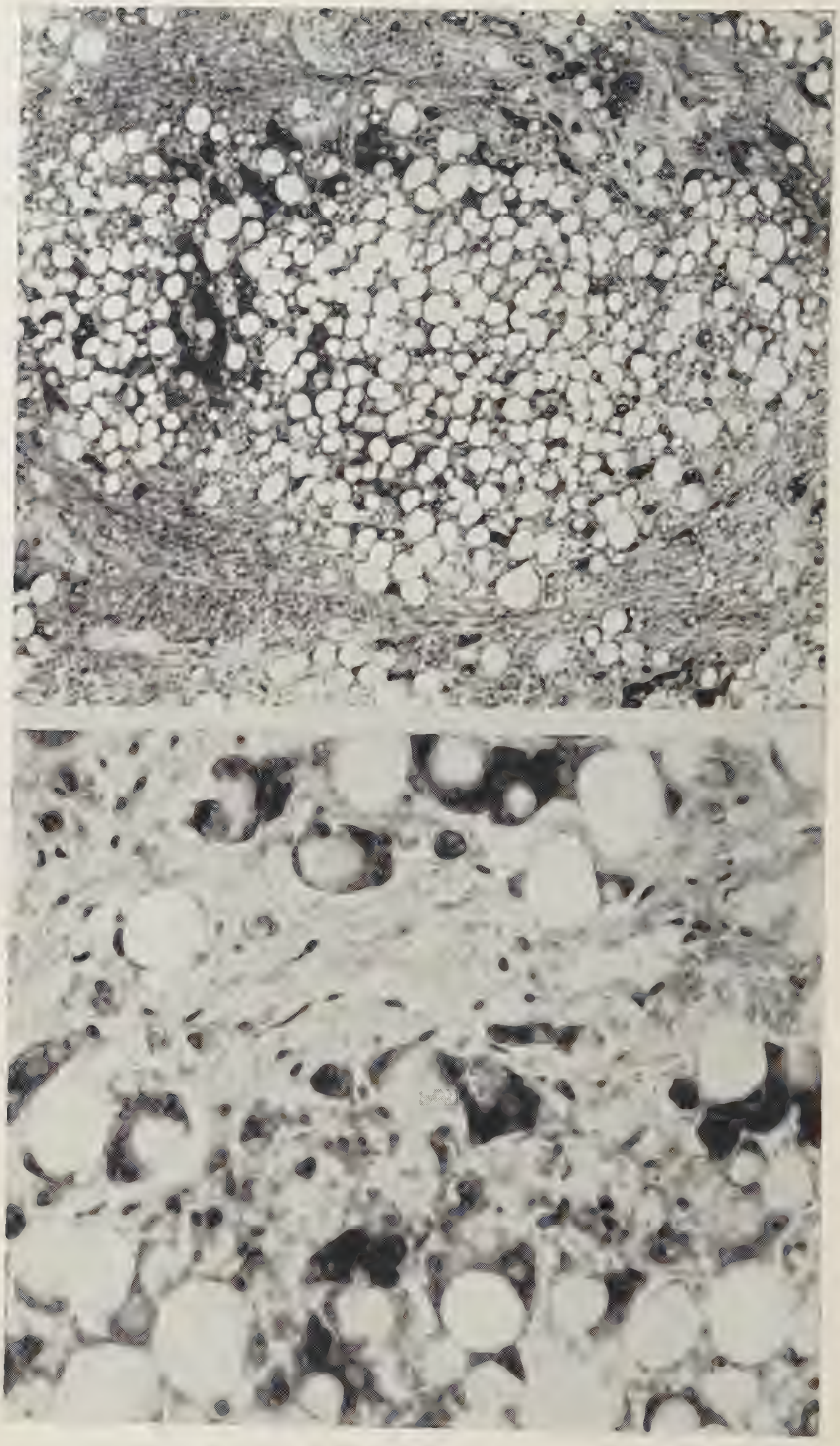
tration of alcohol was begun and the diet changed to approximately 30 g of lean meat. Ten cc of 22.5% alcohol per kilo were administered twice daily but the amount was varied somewhat with the response of the dog. The animals became comatose as a rule within an hour after the alcohol administration. The period of alcohol feeding was not unduly prolonged, 4-7 days of alcohol feeding being alternated with a similar period of fat feeding.

At various intervals after the alcohol treatments were begun, the animals died or were sacrificed and the tissues subjected to histological examination. Sixteen alcohol-treated dogs have so far been studied and the liver findings on these may be summarized as follows: 1. Excessively fatty livers were present in all animals, which resembled those found in chronic severe alcoholism in man. 2. A number of these fatty livers were greenish in color and had intrahepatic obstruction. Atrophy of liver cells was found at the periphery and in these cells hyaline degeneration was observed. 3. Four of the 16 animals showed definite cirrhosis, moderate in degree, but in all ways resembling that found in early fatty cirrhosis in the liver of man.

A typical protocol follows:

Dog F14 weighed 13.3 kilos at the start of the experiment, December 6, 1937. On this day the feeding of the high fat diet was begun and continued until January 10, 1938, at which time it weighed 14.6 kilos. The alcohol feeding was now started and the diet changed to the high protein diet which was supplemented with salts and vitamin sources. Approximately 260 cc of 22.5% alcohol were administered daily and this was increased slowly to 320 cc. In the course of 3 weeks the weight increased to 15.2 kilos, when anorexia developed. For the next 3 weeks alcohol administration was intermittent, partly because its effect appeared to be greater, and partly because of the poor condition of the animal. In another 3 weeks the weight dropped to 13.5 kilos and alcohol was omitted from the diet for several periods, the longest being 7 days. The alcohol kept the animal drunk much of the time and part of the time so sick that he vomited frequently. Ten days before death it was noted that the 2 large superficial epigastric veins on the lower abdomen were markedly dilated. The animal died March 21, 1938, *i.e.,* 106 days after the beginning of the experiment.

Autopsy: Weight 13.5 kilos, weight of liver 980 g. There was no ascites but the intestinal tract was filled with bloody material. The liver was mottled in appearance wth very large lobules, some of which measured 3 mm in diameter. Fatty and fibrous streaks ran through the liver which was quite friable. Blocks were taken for



FIGS. 1 AND 2.

Low power photomicrograph showing a representative liver lobule surrounded by proliferating fibrous tissue, and (below) higher power showing fatty and coagulative degeneration of liver cells, atrophy of cells, and fibroblastic reaction.

histological examination and the remainder ground up for fat estimation. (Total fatty acid, 30%.)

Microscopic Description: The liver shows extensive fatty infiltration This is central in location, although scattered patches are present in different portions of some lobules. Many cells which do not contain much fat are undergoing some rather rapid degeneration of a coagulative hyaline type. Around the periphery of many lobules, cells have become atrophied and have been caught in a meshwork of proliferating fibrous tissue. This has proliferated around lobules forming a characteristic retaining wall. In other lobules it ramifies throughout, breaking the normal lobule up into several smaller lobules. This is better seen in phosphotungstic acid hemotoxylin sections where the picture of cirrhosis of the liver is reproduced. Rather wide bands of fibrous tissue surround the lobules and pass through lobules to connect others and form islands of fatty liver. The picture is that of a well advanced fatty cirrhosis such as can be duplicated by many similar cases called alcoholic cirrhosis in man. (Figs. 1 and 2.)

10204

Pseudopregnancies from Electrical Stimulation of the Cervix in the Diestrum.*

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The artificial induction of a pseudopregnant state in the rat by means of electrical or mechanical stimulation of the cervix uteri is a common laboratory procedure. It has long been supposed that these stimuli to be effective must be applied at or near the time when the female is in a sexually receptive mood (*i. e.*, late proestrus or estrus). We wish to report some observations on the application of an electrical stimulus to the cervix of adult female rats during the diestrous phase of the cycle.

The cervix was exposed with a speculum and the electrodes, spaced at 2 mm, were applied at any place on the body of the cervix. The stimulus, a faradic current taken from an induction coil, was moderately intense and always produced convulsive contractions in the hind quarters The stimulus was applied continuously over a period of 5 to 10 sec.

* Aided by a grant from the Rockefeller Foundation.

In a group of 26 virgin females known to be having regular 4 to 5 day cycles 13 were stimulated on the 1st day of diestrous, and 13 on the second day. The number of animals becoming immediately pseudopregnant, those showing one additional estrus before becoming pseudopregnant and those showing no disturbance of the estrous cycle are listed in Table I.

TABLE I.

No. rats	Day of diestrus cervix stimulated	Number of animals showing: Immediate pseudopreg.	One additional cycle	Uninterrupted cycles
13	1st	7	4	2
13	2nd	2	7	4

The total number of pseudopregnancies obtained in the above groups in which the stimulus was applied during the interval was 20 or 76%. Thirty-six females in late proestrus or estrus were stimulated in the same manner; 31 or 86% became pseudopregnant immediately and the remaining 5 continued to show cycles of normal length. The above figures are all on the basis of a single stimulation for each animal.

Since it was possible by stimulating the cervix in the diestrum to extend the diestrous interval in some instances into a normal pseudopregnancy it seemed probable that this procedure applied during pseudopregnancy might also continue this condition beyond the normal average length of 12 to 14 days. Accordingly 16 females were stimulated every third day beginning on the eighth day of pseudopregnancy. In spite of this treatment the pseudopregnancies were all terminated between the twelfth and sixteenth days. Since stimulation of the cervix during the diestrum may induce a pseudopregnant state one may infer that the corpora lutea are not effectively blocking the neurohumoral mechanism. The reason that this same procedure applied during a pseudopregnancy does not result in a prolongation of that condition is presumably related to the greater degree of activity of the corpora lutea at this time.†

† Astwood finds (unpublished data) that the injection of an adequate dosage of progesterone prior to cervical stimulation will greatly reduce the expected number of pseudopregnancies.

10205 P

Inactivation of Estrogenic Hormone of the Ovary by the Liver.

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Very small proportions of estrogens are recovered in the excretions from animals to which they have been administered. Evidence in support of the theory that the liver is responsible for the inactivation has been obtained by Zondek[1] and many others.[2, 3] Recently, Israel, Meranze, and Johnston[4] found that alphaketohydroxy estrin was rapidly inactivated when added to the perfusate of a heart-lung-liver perfusion system, while no inactivation occurred in perfusion systems consisting of heart and lung, without the liver. These findings suggest that estrin, in the quantity liberated into the blood stream by the ovaries, is inactivated when it reaches the liver.

In order to test this theory, homotransplants of ovaries were made to the mesenteries of a group of rats. The venous drainage was such that any secretion produced by the transplants must pass through the liver before reaching the organs (uterus, vagina, pituitary) upon which the effect of the hormone could be determined. Homotransplants of the ovaries to the axillary region in another group of rats served as controls.

Microscopic sections revealed that the transplants grew well at both sites. Those rats whose ovaries had been transplanted to the axilla resumed their cycles in from 8 to 20 days after the operation. On the other hand, animals whose ovaries had been transplanted to the mesentery showed no evidence in the vaginal smear of coming into heat after 40 days. The pituitaries of 4 of the latter group of animals, assayed by the Lauson, Heller, Sevringhaus technic,[5] were of the potency order of the pituitaries of castrates, in spite of the fact that microscopic sections of the transplants revealed functional ovarian tissue with large follicles and corpora lutea. In 12 of the animals mesenteric transplants of the ovaries were retransplanted to the axillary region. Ten of these animals survived the operation,

1 Zondek, B., *Skand. Arch. f. Phys.*, 1934, **70**, 133.

2 Silberstein, F., Molnar, K., and Engel, P., *Klin. Wchnschr.*, 1933, **12**, 1694.

3 Rondoni, P., Carminati, V., and Corbellini, A., *Z. fur Physiol. Chem.*, 1934, **241**, 71.

4 Israel, S. L., Meranze, D. R., and Johnston, C. G., *Am. J. Med. Sc.*, 1937, **194**, 835.

5 Lauson, H. D., Heller, C. G., and Sevringhaus, E. L., *Am. J. Phys.*, 1938, **121**, 364.

and in all but one estrus reappeared in from 8 to 20 days after retransplantation.

It is evident that the failure to maintain cycles is not due to inhibition of ovarian ability to ripen follicles and form corpora lutea, but to an inhibitory effect on ovarian products depending on the circulatory path from ovary to pituitary or uterus.

These findings serve to explain the marked difference in effectiveness of a given dose of estrogen given orally when compared with parenteral injection. Other applications to clinical problems are not difficult to find.

Further work on the rôle of the liver in estrogen inactivation is being carried on.

10206 P

Eclampsia-like Syndrome Occurring in Pregnant Dogs and Rabbits Following Renal Artery Constriction.

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Liver lesions in the experimental animal resembling those seen in eclampsia have been reported by several investigators, and associated renal injury has been noted following the injection of Vasopressin.[1]

Greene[2] has reported spontaneous eclampsia occurring in the rabbit, demonstrating liver lesions in a large number of animals. The spontaneous disease in sheep and guinea pigs has also been reported. In no instance, to our knowledge, has anything comparable to the spontaneous eclamptic complex with its anatomical lesions been produced experimentally.

We wish to record our observations on the rapidly fatal course and pathological findings in pregnant dogs and pregnant rabbits following constriction of the renal arteries. The suggestive significance of pregnancy as a factor in the fatal effect of renal arterial constriction is emphasized by the remarkable rapid recovery of animals if they are delivered.

Eight pregnant mongrel dogs from the stock colony were used for the experimental procedure. All animals were in good condition at

[1] Byrom, F. B., *J. Path. and Bact.*, 1937, **45**, 1.

[2] Greene, H. S. N., *J. Exp. Med.*, 1937, **65**, 809.

the beginning of the experiment and were maintained in the animal quarters on routine stock diet.

Renal artery clamps modeled after the Goldblatt[3] clamp were applied in such a manner as to produce minimal to moderate constriction of the renal arteries in the pregnant animals. A single renal artery clamp was applied on about the 15th and 20th day before the probable date of delivery in 2 pregnant dogs. The second clamp was applied approximately 10 days later. In 6 pregnant dogs 2 renal artery clamps were applied simultaneously, 2 to 30 days before the expected date of delivery. Periodic blood pressure determinations were obtained by direct arterial puncture. Qualitative urinary examination and blood determinations for N. P. N. and uric acid were made at intervals. Complete autopsies were performed immediately after death except in 2 instances in which there was a delay of more than 4 hours. Two dogs were sacrificed by administration of illuminating gas through inhalation.

Five non-pregnant female and 2 male dogs were used for the control series. Both renal arteries were moderately or severely constricted in these animals, but otherwise they were treated as the animals in the experimental group.

The significant limitation of survival time in the pregnant animals is noteworthy. Within 48 to 120 hours following constriction of the renal arteries the pregnant dogs developed weakness, lassitude, coma, and convulsions, and all exhibited hypertension, hematuria, albuminuria, and nitrogen retention. Death occurred in 5 pregnant animals in from 5 to 15 days, and 2 were sacrificed after 4 and 7 days.

In 2 of the pregnant animals operation was followed by the rapidly progressing toxic signs and symptoms which proved to be characteristic of these pregnant animals. One animal delivered 24 hours later with subsequent rapid improvement, and the animal is now living and well after 8 months with a sustained hypertension. The other animal delivered 5 stillborn and macerated fetuses 48 hours after operation followed by rapid improvement and with all the appearances of ensuing well-being. N. P. N. was 29 mg per 100 cc and uric acid was 1.1 mg per 100 cc 24 hours after delivery. The animal was killed at this time.

Of the control group, despite severe renal artery constriction, one male is living after 11 months, and one female is living after 6 months with both animals maintaining a sustained hypertension. In no instance did we produce in the controls the rapidly fatal clinical

[3] Goldblatt, Lynch, Hanzal, and Summerville, *J. Exp. Med.*, 1934, **59**, 347.

course with minimal arterial constriction such as occurred in the pregnant animals. In 4 control dogs it was necessary to increase the severity of the renal artery constriction at subsequent operations. This group of dogs died in uremia 3 to 30 days following the severe renal artery constriction. These observations suggest that some factor other than simple renal insufficiency is concerned in the production of the rapid and fatal course noted in the pregnant animal.

The significant autopsy findings in the 7 pregnant dogs with constricted renal arteries were evidences of terminal acute cardiac failure with pulmonary edema (with infarction in some instances), hemorrhage and infarction in the myocardium, and scattered hemorrhages in the gastro-intestinal tract. The kidneys showed changes characteristic of renal ischemia with lobular renal necrosis where renal artery branches had been occluded. Alterations noted in tissues of one animal were impossible to interpret microscopically due to terminal invasion by gas bacilli. Of the remaining 6 pregnant animals, all showed suggestively significant widespread liver lesions. In 2 pregnant animals conspicuous large areas of coagulation necrosis were present with hemorrhage, laked red cells and fibrin. These lesions imitate closely those described by many as typical of human eclampsia and which are known to consist primarily of periportal necrosis with hemorrhage and periportal "laking". In addition to this more specific lesion there were in the pregnant animals all degrees of liver injury varying from simple periportal cloudy swelling, dilatation of sinusoids, and hemorrhage to occasional periportal necrosis and even more conspicuous focal necrosis. These lesions appear to have developed in a manner comparable to that which obtains in human eclampsia and indeed they resemble the hepatic lesions of this disease.

In the control animals small hemorrhages in the myocardium were occasionally seen. The liver showed only an intense central congestion. The kidneys and lungs did not differ from those of the experimental animals.

In another experiment with groups of pregnant and control rabbits and in which a similar but modified technic was used our results were remarkably comparable to those recorded in the dog experiments. In pregnant rabbits we noted a uniformly characteristic response. Death occurred usually within 48 to 120 hours and autopsy showed lesions involving kidneys and liver comparable to those described in spontaneous eclampsia of rabbits (Greene[2]).

These preliminary observations indicate that experimentally produced renal ischemia or renal injury in pregnant dogs and pregnant

rabbits results in an eclampsia-like syndrome, characterized by a rapidly fatal clinical course and significant pathological lesions in the liver and kidney.

We wish to suggest the probability of a correlation between the physiological and pathological processes underlying these observations and the mechanism of human eclampsia. These and further observations will be reported more fully when experiments now under way have been completed.

10207 P

Further Observations on the L Organism of Klieneberger.

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The L organism which Klieneberger[1] isolated from the cultures of *Streptobacillus multiformis* and more recently directly from pathological lesions of rats presents many characteristics which are not observed in bacteria. It has a marked similarity to a group of microörganisms, the main representative of which is the causative agent of pleuropneumonia bovis. The relation of this group to the well characterized classes of microörganisms is obscure. The L organism starts to grow in very small units which pass readily through a coarse bacterial filter. Later the small forms develop into large yeast-like bodies. Probably the small bodies are reproduced by the disintegration of the large forms. In all stages of development the organism is very fragile and its form can be seen only by using special technic. Since pleuropneumonia-like organisms occur in different animals[2] and probably also free in nature,[3] their occurrence in rats is not unexpected. However, it is very surprising to find such organisms regularly in the cultures of a bacillus.

Klieneberger regards the connection between the L organism and

[1] Klieneberger, E., *J. Path. Bact.*, 1935, **40**, 93; 1936, **42**, 587; *J. Hyg.*, 1938, **38**, 458; Klieneberger, E., and Steabben, D. B., *J. Hyg.*, 1937, **37**, 143.

[2] Shoentensack, H. M., *Kitasato Arch. Exp. Med.*, 1934, **11**, 277; 1936, **13**, 175 and 269) (Quoted after Klieneberger).

[3] Seiffert, G., *Zbl. Bakt. I. O.*, 1937, **139**, 337; Oerskov, J., *Zbl. Bakt. I. O.*, 1938, **141**, 229.

streptobacillus as a symbiosis. The two main arguments in favor of this supposition are: (1) the striking differences between the L organism and the bacillus; (2) the constancy of the properties of the L organism which in several years of cultivation never reverted to bacterial forms. The occurrence of L organisms without bacteria in animal lesions and the observation that the L organism forms mixed cultures with bacillus tetanus and certain other bacteria, give further support to the hypothesis that the L organism is a symbiont of the bacillus.

The observations which are here reported do not agree with this supposition. In the colonies of the streptobacilli the characteristic swollen forms do not develop as a separate growth admixed with the bacteria but are produced by the swelling up and transformation of the streptobacilli themselves. These large forms persist for a while or soon degenerate, but neither in the original cultures nor in transplants do they show any signs of multiplication. A transplant from a colony consisting mostly of the large forms gives a pure culture of the bacilli. The L organisms start to grow in the bacterial colonies after 48 hours or more of incubation in the form of a few secondary colonies attached to and partly embedded in the agar—and can be easily transplanted from these. The appearance of the L organism in transplantable form in the streptobacillus colonies is similar to the appearance of a colony variant.

Recent observations made with a freshly isolated strain of the streptobacillus strongly suggest that the L organism is actually a variant or growth form of the streptobacillus. The L organism was isolated from the secondary colonies of the streptobacillus. From the second transplant on, no streptobacilli grew in the cultures of the L organism on agar. This same strain of L organism was used for morphological studies and daily for 2 months numerous microscopical preparations were made from the cultures. No bacillary forms were visible in these preparations. As the bacillus grows faster and produces larger colonies than the L organism it is improbable that it would persist in latent form together with the L organism. However, on 3 different occasions small blocks of agar covered with colonies of L organism were put into nutrient broth containing boiled blood and ascitic fluid. For the first few days the L organism was present in the broth in pure culture, but after 3 days or more streptobacilli invariably appeared. Before the third experiment the L organism was consecutively transplanted 5 times from isolated colonies. Four of 6 broth tubes inoculated from this purified culture of L organism were found to contain streptobacilli, while 2 were contaminated during the successive transplants.

It should be mentioned that formerly strains of L organism directly isolated from the lungs of rats were maintained in the same kind of broth, but in these cultures the streptobacilli never appeared. Klieneberger recently pointed out that the strains directly isolated from the lesions are different both in their morphology and serological specificity from the strains isolated from the streptobacilli. The latter strains are serologically similar to the streptobacilli. Thus, the failure to cultivate the streptobacilli from the strains directly cultured from the lesions is not unexpected. Furthermore, the marked difference between the 2 groups of L strains in itself is rather against the symbiosis hypothesis. It is improbable that the symbiosis would so profoundly alter the properties of the strains.

The occurrence of such remarkable variation as the L organism in bacterial cultures is of considerable importance. For this reason caution is necessary in interpreting the observation. In a subsequent report observations will be presented and discussed showing that various groups of bacteria show more or less similar phenomena, thus supporting the conclusion that the L organism is a variant of the bacillus rather than a symbiont.

10208 P

Effect of Acute Anoxia Produced by Breathing Nitrogen, on the Course of Schizophrenia.

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Previous work has led us to believe that a common factor in the hypoglycemic and metrazol treatments for schizophrenia is a diminished cerebral metabolism. The mechanism for the production of the decreased metabolism is different for each treatment. During hypoglycemia the cerebral metabolism diminishes because the brain is deprived of its chief substrate, glucose.[1] After the injection of metrazol, on the other hand, the inhibition of cerebral metabolism is evidently due to an acute anoxemia induced by the severity of the convulsions and the temporary arrest of respiration.[2]

1 Himwich, H. E., Bowman, K. M., Wortis, J., and Fazekas, J. F., *Science*, 1937, **86**, 271.

2 Himwich, H. E., Bowman, K. M., Fazekas, J. F., and Orenstein, L. L., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 359.

If the beneficial results following metrazol therapy are dependent upon decreased cerebral metabolism resulting from acute anoxia, then anoxia of a similar degree, produced by any means should be equally effective. It occurred to us that acute anoxemia might be produced under safer and more controllable conditions by having the patients breathe for short periods an atmosphere in which the nitrogen was increased at the expense of oxygen. By this method it is possibe not only to control the degree of anoxemia produced but also to terminate the bout immediately in the event of untoward reaction.

The production of acute anoxemia was under the direction of one of us, F.A.D.A., a trained anesthetist. The apparatus used included a tight fitting face mask with exhalation valve such as is used in anesthesia, a canister of soda lime and a 5-liter breathing bag connected in series. The apparatus was filled with pure oxygen, adjusted to the patient's face and after a few breaths, nitrogen was run into the mask gradually, displacing the oxygen. Unlike previous attempts of other workers the anoxia was intense. At the height of the bout the patient was respiring almost pure nitrogen. Carbon dioxide did not accumulate but was absorbed by soda lime, thus minimizing the patient's discomfort during the treatment.

As anoxemia became progressively more severe the usual signs, respiratory stimulation, tachycardia, rising blood pressure, cyanosis, neurological changes, *i. e.,* opisthotonos, convulsive jerkings and extensor and torsion spasms appeared. At the point when it was felt that more severe or prolonged anoxemia would jeopardize the patient's safety the bout was terminated suddenly by introducing pure oxygen into the mask. Consciousness returned immediately and with the exception of occasional headache there have been no complicating sequelæ.

Five patients with schizophrenia were so treated 3 times a week; 2 for approximately 4 months and one, 2 months and 2 more for about a month. One of the 3 patients who received the treatment longest has been discharged in full remission, and is still feeling well being entirely free from delusional formations. The other 2 are improved though still hospitalized. One of the patients receiving the treatment for only one month has begun to show improvement. An interesting phenomenon—all 3 patients of the first group were more disturbed, restless and apprehensive when due to circumstances treatment was stopped for a week. The delusions and hallucinations reappeared in one patient and persecutary ideas returned in another patient during the interval. These symptoms subsided when treatment was reëstablished.

Since our therapy simulates metrazol treatments it may be well to compare the two. The present procedure insures a greater depletion of the oxygen saturation of the blood than does the metrazol. With metrazol the hemoglobin saturation may decrease to 40%, while with our treatment the hemoglobin saturation was 30% or lower, going down to 15%. Despite a degree of anoxemia more profound than that attained with that of metrazol the patient is nevertheless under control of the anesthetist, thus insuring greater safety.

Though our experience is limited with this treatment of schizophrenia, the results are encouraging. Many more data must be accumulated before conclusions can be drawn. Nevertheless, the results with anoxia do suggest that a profound diminution of cerebral metabolism produced by this technic exerts favorable effect in the course of schizophrenia.

We are grateful to J. F. Fazekas, S. J. Martin, H. E. Doudna, and H. C. Slocum for their coöperation in this investigation.

10209 P

Causes of the Discontinuity of Growth of Fibroblasts Cultivated in Embryo-Juice.

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Fibroblasts that are cultivated according to the flask-technic in a plasma-coagulum with embryo-juice as nutrient fluid do not proliferate continuously. Generally after 2 or 2½ weeks' cultivation the cells stop multiplying, although only a small portion of the coagulum is covered with tissue. If a part of the tissue is transferred to a new coagulum, growth is resumed. If it is not so transferred the cells degenerate. Investigators who have attempted to find the cause for this cessation of growth and subsequent degeneration of the cells have attributed it either to changes that take place in the physical structure of the plasma-coagulum, or to the accumulation of toxic products therein.[1] The experiments reported here were

[1] Ephrussi, B., *Arch. d'Anat. Micros.*, 1933, **29**, 95; Fischer, A., *Cytologia*, 1930, **1**, 217, and *Virchow's Arch. Path. Anat. u. Physiol.*, 1930, **279**, 94; Mayer, E., *Arch. f. Entwich. Mech.*, 1933, **130**, 382; *Compt. Rend. Soc. Biol.*, 1935, **119**, 422; Olivo, O. M., *Arch. f. Exp. Zellforsch.*, 1931, **11**, 261.

designed to test another hypothesis, namely, that it might be the removal of serum from the coagulum that is responsible for the cessation of growth. Or, to express it in another way, it might be an inadequacy in the food supplied.

In the first experiment 2 fragments of tissue from a 23-year-old* strain of chick-heart fibroblasts were embedded in a flask 3½ cm in diameter in a coagulum containing 33% plasma. They were then cultivated for 9 days in 33% embryo-juice.† By this time their initially rapid growth had already decreased to a noticeable degree. Two drops of chicken-serum were then given every 48 hours in addition to the embryo-juice that had previously been supplied. The cultures immediately resumed active growth. But, after 9 more days of cultivation their growth-rate diminished again. The concentration of embryo-juice was then increased to 66%, and serum was given as before. Again, active growth was resumed. This time it continued until the edge of the colony reached the vertical side of the flask.

These results indicate that two factors are involved in the cessation of growth: (1) that serum is needed as well as embryo-juice as a nutrient for the cells, and (2) that the embryo-juice must be supplied in higher concentration than that usually given. To test these hypotheses further, 3 experiments were made. Cultures of chick-heart fibroblasts were divided into 2 equal parts. These were embedded in separate flasks, 3½ cm in diameter, in coagula containing 33% chicken-plasma. A single fragment of tissue was placed in each flask as near the center of the coagulum as possible. In the first experiment one-half of the original culture was cultivated in 33% embryo-juice, the other in 33% embryo-juice to which serum at either 4 or 8% concentration was added. In the second experiment one-half of the tissue was cultivated in 66% embryo-juice, the other in a mixture of 66% embryo-juice and 8% serum. And in the third experiment serum an 8% concentration was given to both tissues but one was supplied with embryo-juice at 33% and the other with embryo-juice at 66% concentration. In each case, a few days were allowed to elapse at the beginning of the experiment before the serum was added to the nutrient fluid, since there was at this time more than an adequate supply of serum in the coagulum. And, of course, each experiment was repeated a number of times.

In the first experiment the colony cultivated in 33% embryo-juice

* Experiments made in 1934 to 1935, but not published.

† Made by extracting one volume of pulp from 9-day embryos with 3 volumes of Tyrode's solution.

stopped its active proliferation after 2 weeks, *i. e.*, at just about the time that all of the serum was removed from the coagulum. The sister-culture which was given serum as well as embryo-juice proliferated for a longer time, and grew to be much thicker, but did not fill the flask. In the second experiment the colony that was cultivated in 66% embryo-juice grew at an exceedingly active rate for 14 days, but, then, while still proliferating actively suddenly liquefied the coagulum. The sister-colony that received serum with the embryo-juice continued its proliferation and eventually covered the entire coagulum, forming a colony 3¼ to 3½ cm in diameter, and many cell-layers thick. In the third experiment the colony receiving serum and embryo-juice at 33% concentration grew much more slowly than the sister-colony and stopped growing before the coagulum was covered with tissue, while the one which received serum and embryo-juice at 66% concentration grew as long as cultivation was continued, *i. e.*, until the colony reached the vertical side of the flask. In one of these experiments in which a strain of heart-tissue in its sixth passage *in vitro* was used the tissue covered the entire coagulum in 17 days. In other experiments continuous growth was observed for a period of 30 to 36 days.

It would seem, therefore, that the cessation of growth observed under the usual conditions of cultivation is not due to the change that occurs in the structure of the coagulum. Neither is it due to the accumulation of toxic substances in that coagulum, but is caused by the removal of serum which is required for proliferation in addition to embryo-juice.‡ Moreover, after the colony has reached a certain size a higher concentration of embryo-juice than that which is usually given must be supplied.

‡ Hanging-drop cultures of fibroblasts in a medium made by extracting embryo-pulp with serum have been reported by des Ligneris, M. J. A., *Arch. Exp. Zellforsch.*, 1936, **18**, 442, but the object of his work and the results obtained were quite different from those reported here.

10210

A Synergism of Physostigmine and Strychnine.

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The experiments of Feldberg, Minz and Tsudzimura[1] on the mechanism of the nervous discharge of epinephrine suggest the possibility of influencing the liberation of this hormone through the synergistic action of appropriate drugs If, as outlined in their theory, splanchnic stimulation results in the production of acetylcholine which in turn stimulates the adrenal medulla to discharge epinephrine, drugs capable of effecting splanchnic stimulation will act synergistically with drugs capable of prolonging the action of acetylcholine. Employing strychnine[2] and physostigmine,[3] as representatives of the aforementioned drug types, we have demonstrated in unanesthetized rats a synergistic hyperglycemic action, presumably due to the liberation of epinephrine since the effect disappeared when the adrenals were demedullated. In the quantities employed the drugs did not produce objectionable systemic effects.

Methods. The data were obtained by superimposing the actions of physostigmine and strychnine on a glucose tolerance test[4] standardized for the rat. Only male rats of the Wistar strain ranging in age from 93 to 219 days were used. The diet was uniform.

Fifteen to 16 hours subsequent to the removal of the food, the test glucose, 3.5 g per kg in a 10% solution, was injected intraperitoneally. Blood samples, sufficient for measuring 0.2 cc were milked from the tail before the glucose injection and ½, 1, 2, 3, and 5 hours after.

"True" sugar was determined by the Shaffer-Hartmann-Somogyi method[5] on blood filtrates prepared by the zinc hydroxide precipitation procedure.[6] Newcomer hemoglobin determinations indicated that the blood concentration changes were not sufficient to justify a correction of the blood sugar values.

Test routine. The data were obtained on 22 rats each subjected to the glucose tolerance test under the following conditions: (1) no

1 Feldberg, W., Minz, B., and Tsudzimura, H., *J. Physiol.*, 1934, **81**, 286.

2 Stewart, G. N., and Rogoff, J. M., *J. Pharm. and Exp. Ther.*, 1919, **13**, 95.

3 Stedman, E., and Stedman, E., *Biochem. J.*, 1931, **25**, 1147.

4 Cole, V. V., Harned, B. K., *Endocrinology*, 1938, **23**, 318.

5 Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

6 Somogyi, M., *J. Biol. Chem.*, 1930, **86**, 655.

drug; (2) with strychnine; (3) with physostigmine; (4) with physostigmine and strychnine. Subsequently, the adrenals were demedullated and procedures (1), (3), and (4) repeated. The routine was limited to 6 tolerance tests per day; 2 of the rats received strychnine, 2 physostigmine, and 2 strychnine and physostigmine. Although 6 controls (no drug) were made on a single day, they were distributed at regular intervals throughout the experimental period. According to data of the authors,[4] the glucose tolerance of Wistar rats is constant over a much larger age range than that involved in the present study. Tests on individual animals were spaced 10 to 14 days apart. Immediately after the 1 hour blood had been taken the drug was injected subcutaneously and the difference between the blood sugar values obtained with and without the drug measured the effect of the drug. The ½ hour and the 1 hour bloods served as added controls on the condition of the animals.

Throughout the experiments the drugs and the glucose were dissolved in distilled water immediately before their injection. The physostigmine and the strychnine were administered in the following concentrations and quantities: (1) strychnine sulfate (U.S.P. Merck), 0.52 mg per kg in a 0.035% solution; (2) physostigmine salicylate (U.S.P. Merck), 0.10 mg per kg in a 0.010% solution. The physostigmine used in the experiments was obtained from one ampoule, kept dry in a desiccator.

Results. In the concentrations employed the drugs rarely gave external evidence of activity. The quantity of physostigmine was selected because it appeared to be the largest amount just failing to produce salivation and of the 44 injections of this drug alone and in conjunction with strychnine, salivation was observed in only 2 instances. There was no cathartic action, no muscular twitching, and, according to palpations at significant intervals, no change in heart rate. Comparing the blood sugar values obtained after physostigmine with those of the corresponding control periods (Table I), the elevation produced by the drug amounted to 41 mg % on the first hour, 35 mg % on the second hour, and 20 mg % on the fourth hour.

The amount of strychnine administered was approximately the smallest quantity producing an elevation of the blood sugar. By the fourth hour subsequent to the injection, the strychnine effect apparently had disappeared, but on the first and second hours the elevation averaged 6 mg %. The chance that this figure is significant is 9 to 1. Probably, the synergism would have been magnified if a larger quantity of strychnine had been chosen.

TABLE I.

Effect of Physostigmine and Strychnine on Glucose Tolerance of Normal and Demedullated Rats.

	No. of animals	Fasting*	Hr after glucose injection: ½	1	2	3	5
			Hr after drug injection:		1	2	4
		"True" blood sugar in terms of glucose per 100 cc. of blood					
		mg	mg	mg	mg	mg	mg
No drug	22	66 ± 1.0	170 ± 3.2	136 ± 2.7	113 ± 1.4	112 ± 0.8	110 ± 0.8
Strychnine	21	66 ± 1.0	172 ± 3.3	134 ± 2.2	119 ± 2.0	118 ± 2.3	110 ± 2.6
Physostigmine	22	64 ± 0.7	171 ± 3.2	137 ± 2.8	154 ± 3.4	147 ± 2.9	130 ± 5.0
Physostigmine and strychnine	22	66 ± 0.8	171 ± 3.2	135 ± 2.9	174 ± 5.1	173 ± 5.9	134 ± 4.1
		After adrenal demedullation					
No drug	18	67 ± 0.9	171 ± 2.8	135 ± 3.1	107 ± 3.1	92 ± 3.1	89 ± 2.7
Physostigmine	18	71 ± 0.7	166 ± 3.8	121 ± 2.7	108 ± 2.0	97 ± 2.4	68 ± 2.4
Physostigmine and strychnine	18	68 ± 0.9	157 ± 3.4	118 ± 1.7	107 ± 2.0	85 ± 2.8	60 ± 4.0

*Food withdrawn 15 hours before control blood.

Glucose was administered intraperitoneally, 3.50 g per kg of body weight. The drugs were injected subcutaneously immediately after the 1-hour bloods. When 2 drugs were administered, they were given separately. The drug dosages per kg were: physostigmine salicylate, 1.0 cc of a 0.01% solution; strychnine sulfate, 1.5 cc of a 0.035% solution.

When the physostigmine and strychnine were injected simultaneously the synergistic rise in the blood sugar amounted to 14 mg % on the first hour, 20 mg % on the second hour, and 4 mg % on the fourth hour (Fig. 1). On a percentage basis the synergistic action increased the hyperglycemic effect of the strychnine 230% on the first hour, and 330% on the second hour, or it increased the summed effects of strychnine and physostigmine 29% and 47% respectively on the first and second hours. While the data for the fourth hour subsequent to the drug injection do not permit a definite conclusion, they contain the suggestion that physostigmine has demonstrated a strychnine action not detectable in its absence.

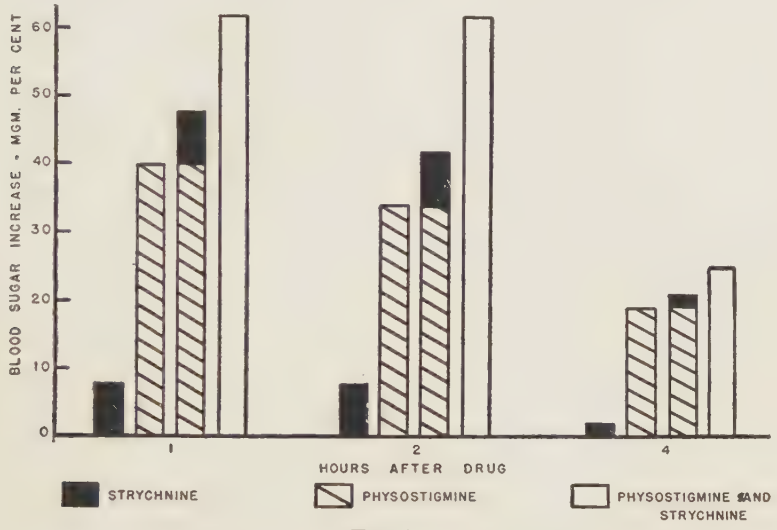

FIG. 1.

Hyperglycemic Synergism of Physostigmine and Strychnine.

The drugs were administered subcutaneously in the following dosages per kg: physostigmine salicylate, 1.0 cc of a 0.01% solution; strychnine sulfate, 1.5 cc of a 0.035% solution.

Supplementing the statistical analysis of the mean values given in Table I, the range of the blood sugar variations provides additional evidence of the synergism. It is significant that, although the boundaries for the range of the blood sugar variations of strychnine alone closely approximate those of the control, without drugs, the boundaries for the combination of strychnine and physostigmine are impressively above the corresponding limits of physostigmine alone.

The quantities of physostigmine and strychnine employed are not considered optimal for the synergistic demonstration since they were selected as representing definite hyperglycemic doses free from objectionable side reactions in the rat.

After demedullation of the adrenals by Evans' technic[7] the hyperglycemic action of the drugs almost, if not entirely, disappeared (Table I). The effect of the drugs may be evaluated by comparing the blood sugar averages with and without the drugs at a given time or by comparing the progressive changes in the control series with those in the drug series. With the unoperated rats the 2 methods give identical results but with the demedullated animals the first method shows no hyperglycemia from the drugs while the second method indicates a questionable rise in the blood sugar. Using the second method, with the value of the blood sugar immediately before the drug injection as the point of reference, physostigmine and strychnine produce after one hour a hyperglycemia of 18 mg % in the demedullated rats as compared with 62 mg % in the unoperated animals and after 2 hours, 10 mg % in the operated but 62 mg % in the unoperated. Four hours subsequent to the drug injections the demedullated rats showed a hypoglycemia of 12 mg % while the unoperated rats showed a hyperglycemia of 25 mg %. Control experiments on the demedullated rats were made 25 and 60 days after demedullation. The glucose tolerance remained essentially unchanged. During the interval the drugs were administered. The quantitative absorption of the sugar from the intraperitoneal injection did not differ from that in the unoperated animals.

Conclusions. Physostigmine and strychnine administered to normal rats in doses of 0.10 and 0.52 mg per kg respectively, exerted a statistically significant, hyperglycemic synergism. The synergistic action increased the mean of the maximal hyperglycemia of strychnine 330% and the sum of the means of the maximal actions of physostigmine and strychnine 47%. The quantities of the drugs employed did not produce objectionable systemic effects. After demedullation of the adrenals the drugs lost their hyperglycemic action.

[7] Evans, G., *Am. J. Physiol.*, 1936, **114**, 297.

10211

Saturation of Serum with CO_2. A Simple Test for Hyperglobulinemia.*

HANS NORBERT NAUMANN.† (Introduced by C. H. Greene.)

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Ray[1] described the development of cloudiness or flocculation on dilution of the blood with distilled water in cases of kala-azar. Normal blood gives a clear solution on such dilution. An improvement of this test was introduced by Sia,[2] who used measured amounts of blood and distilled water and distinguished different degrees of reaction. The application of the water test in Bilharziasis was described by Salah.[3] The general applicability of this test in conditions of hyperproteinemia was pointed out by Bing.[4] A positive water test in Stiil's disease was described recently by Taussig.[5]

The test is based on the flocculation of euglobulin, the least soluble globulin fraction. Generally it is positive within an hour in diseases associated with high globulin concentrations such as kala-azar, lymphogranuloma-inguinale, multiple myeloma, etc. However, if the concentration of globulin ranges between 3 and 4%, a positive test is obtained only after standing for a considerable time. In these cases the test can be rendered more sensitive and the reaction accelerated by saturating the diluted serum with carbon dioxide of low tension or alveolar air. The flocculating tendency is increased by the lowering of the pH toward the isoelectric point of globulin, pH 5.2. The actual acidity produced under various conditions of dilution and carbon dioxide saturation, as measured with the quinhydrone electrode is given in Table I. The figures of 6.12 and 5.95 are approximately in agreement with the pH of 5.8 reported by Doladihle and Morel[6] in 1:17 diluted serum after saturation with carbon dioxide gas.

* I wish to thank the physicians at Kings County Hospital for the use of their cases in this study and particularly Dr. Carl H. Greene for his help and interest in this work.

† Aided by a grant from the Emergency Committee in Aid of Displaced Foreign Physicians.

1 Ray, S., *Ind. Med. Gaz.*, 1921, **56**, 9.

2 Sia, R. H. P., *Chin. Med. J.*, 1924, **38**, 35.

3 Salah, M., *J. Egypt. Med. Assn.*, 1937, **20**.

4 Bing, J., *Act. Med. Scand.*, 1937, **91**, 336.

5 Taussig, A. E., *J. Lab. Clin. Med.*, 1938, **23**, 833.

6 Doladihle, M., and Morel, C., *Compt. Rend. Soc. Biol.*, 1936, **122**, 1210.

TABLE I.
pH of Serum Diluted with Distilled Water and Saturated with Carbon Dioxide of Room Temperature.

	Serum dilutions 1:10	1:100
Without CO_2	7.60	7.75
Saturated with CO_2 of 40 mm Hg (alveolar air)	6.89	6.43
Saturated with CO_2 gas	6.12	5.95

Saturation of diluted serum with carbon dioxide precipitates the euglobulin more or less completely. Myttenaere and Bessemans[7] determined the total globulin in the serum by saturating the 1:10 diluted serum with carbon dioxide. However, they give no figures to demonstrate the accuracy of this method. In 4 cases of multiple myeloma Jacobson[8] found that the precipitate obtained by carbon dioxide saturation contained from 9-24% of total globulin. The present results as shown in Table II corroborate those of Jacobson. It would appear, therefore, that this method yields figures that are too low for the total globulin, so it can not be used as a quantitative test.

TABLE II.
Determination of Protein Precipitated by Carbon Dioxide Gas Saturation. (Micro-Kjeldahl).

Total protein	7.0	7.0	7.2	8.5	8.8
Globulin (21.5% Na_2SO_4)	2.8	2.0	3.8	3.6	4.7
Serum diluted 1:10 saturated with carbon dioxide gas	0.2	0.5	1.1	0.4	0.8
Percentage precipitated by carbon dioxide	7	25	29	11	17

It is of value, however, as a qualitative test for the detection of hyperglobulinemia. For diagnostic purposes saturation with carbon dioxide of 40 mm tension or with alveolar air is preferable to saturation with pure carbon dioxide gas for the latter gives a marked precipitation in normal serum. This 40 mm CO_2 test is performed as follows: 0.1 cc of clear non-hemolyzed serum is placed in a 10:100 mm test tube, 1 cc of distilled water added and the fluid saturated with alveolar air.

Evaluation of the test:

— Slight cloudiness, reflections on test tube observed against source of light sharp, no flocculation in standing.
± Cloudiness, reflections visible, slight deposit on bottom of test tube after 24 hours.
+ Definite cloudiness, reflections unsharp, distinct flocculation after 10-15 minutes.
++ Opaqueness, reflections not visible, coarse flocculation after 5-10 min.

[7] Myttenaere, F., and Bessemans, *Compt. Rend. Soc. Biol.*, 1922, **87**, 800.
[8] Jacobson, B. M., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1257.

The test was compared with the formol-gel, Takata-Ara and Bauer tests in a series of cases in which the albumin and globulin concentration of the serum was quantitatively determined using Minot and Keller's[9] modification of Greenberg's colorimetric technic. The results are given in Table III in which the cases are arranged according to decreasing globulin concentrations.

TABLE III.
Results of Serum Reactions in 25 Cases.

Case No.	Diagnosis	Albumin	Globulin	40 mm CO_2 test	Formol-gel	Takata	Bauer
1 L.D.	Lymphogran. inguin.	3.5	5.1	++	+++	+++	+++
2 A.B.	Hepatic cirrhosis	3.8	4.9	++	+++	+++	+++
3 J.D.	Secondary lues	4.9	4.6	++	++	++	++
4 L.S.	Icterus (unknown origin)	4.4	4.3	++	+++	+++	+++
5 H.M.	Carcinomatosis	3.3	4.0	—	—	±	±
6 W.R.	Pulmonary tuberculosis	3.4	3.7	+	++	++	—
7 R.H.	Pneumonia	4.3	3.5	++	++	±	+
8 L.D.	Secondary lues	4.2	3.4	++	++	+	++
9 G.W.	" "	4.3	3.0	+	±	±	—
10 W.J.	Pulmonary tuberculosis	3.8	2.9	++	+	+++	+++
11 J.C.	" "	4.3	2.8	±	±	+	—
12 J.G.	" "	3.4	2.7	+	—	++	+
13 M.K.	Hepatic cirrhosis	5.8	2.7	+	—	±	+++
14 J.M.	Secondary lues	4.0	2.4	—	±	±	±
15 F.A.	Pneumonia	3.9	2.4	—	—	—	—
16 K.N.	Secondary lues	4.8	2.4	—	±	+	—
17 J.H.	Pneumonia	3.7	2.2	±	—	±	—
18 W.R.	Paget's disease of the bone	4.1	2.1	++	—	+	—
19 J.L.	Secondary lues	5.3	2.1	±	—	—	+
20 A.R.	Pneumonia	4.4	2.0	±	—	—	+
21 A.S.	Pneumonia	4.2	1.9	±	—	±	—
22 E.M.	Paget's disease of the bone	4.4	1.9	—	—	±	—
23 C.D.	Pulmonary tuberculosis	3.9	1.8	—	—	—	±
24 I.G.	Cholecystitis	4.7	1.7	—	—	—	—
24 H.D.	Pneumonia	5.0	1.6	—	—	—	—

It will be seen from the table that in cases Nos. 1-13, with globulin concentrations ranging from 5.1 to 2.7% the 40 mm CO_2 test was ++ or +, in general agreement with the result of the formol-gel, Takata and Bauer tests. A noteworthy exception is case No. 5 with negative CO_2 and formol-gel tests in the presence of a globulin concentration of 4%. On the other hand, cases Nos. 12 and 13 with globulin concentrations of 2.7% and case No. 18 with a globulin concentration of 2.1% gave positive 40 mm CO_2 tests while negative results were obtained with the formol-gel tests. The Takata-Ara test was positive in 2 of these cases and the Bauer test positive in

[9] Minot, A. S., and Keller, M. A., *J. Lab. Clin. Med.*, 1936, **21**, 743.

the third. A negative test in the presence of hyperglobulinemia may be explained by the fact that high globulin concentration is due to an increase in the concentration of the less readily precipitated pseudoglobulin I and II rather than to an increase in the euglobulin fraction. On the other hand, a positive test in the presence of a normal total globulin concentration, may have been caused by a relative increase in the euglobulin fraction. A more detailed discussion of these problems is reserved for another paper.[10]

From the results as presented above it can be concluded that the 40 mm CO_2 test generally is positive, if the globulin concentration of the serum is increased beyond 3% and that the reaction is in accord with the Takata-Ara, Formol-gel, and Bauer tests. As a test for hyperglobulinemia the 40 mm CO_2 test has the advantage of simplicity and speed.

10212 P

Specificity of Kerateine Derivatives.

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In a previous communication,[1] it was shown that species specificity is an individual characteristic of the keratins. The suggestion was offered that the redox state and spatial arrangement of the amino acids cystine and cysteine may be intimately connected with the specificity of these proteins.

In this article it will be shown that the substitution of the hydrogen in the –SH groups of reduced keratin (kerateine) with organic halogen compounds gives the protein a new immunological character dependent on the introduced "determinant group."

Since keratins contain a very high percentage of di-sulfide sulfur (10 to 15% cystine) and are readily reduced to sulphydryl proteins by alkaline thioglycolate, a large number of substituent groups may be introduced into their molecules by reaction with simple organic halogen compounds according to the following formula:

$$A — SH + R — X \rightarrow A — S — R + HX$$

Such a reaction occurs readily in a mildly alkaline environment,[2]

[10] Greene, C. H., and Naumann, H. N., to be published shortly.

[1] Pillemer, L., and Ecker, E. E., *Science*, 1938, **88**, 16.

[2] Goddard, D. R., and Michaelis, L., *J. Biol. Chem.*, 1935, **112**, 361.

and this has been applied to proteins by Mirsky and Anson,[3] and Goddard and Schubert.[4] The method involves a mild treatment of the protein and has the added advantage of being carried out at a pH of 8.

Although Michaelis and Schubert[5] have observed that organic halogens react with amino groups, there was no demonstrable substitution of the amino groups under the conditions employed in these experiments, while complete or near complete substitution of the sulphydryl groups was accomplished.

The derivatives studied were prepared from wool and feather kerateine by reaction with iodo-acetic acid, alpha bromo-propionic acid, alpha bromo-n-butyric acid, alpha bromo-iso-butyric acid, alpha bromo-n-valeric acid, alpha bromo-iso-valeric acid, alpha bromo-n-caproic acid, alpha bromo-ethyl-benzene, and benzyl chloride.

The nomenclature designated by Goddard and Michaelis[2] was adopted, *i. e.,* by reaction with iodo-acetate, alpha-carboxy-methyl kerateine was obtained. The methods used in the preparation of the compounds and the analytical data will be detailed in a forthcoming paper.

The chemical analysis of the derived proteins revealed that the percentages of total nitrogen and sulfur contained in these compounds are similar to the values obtained in the case of the parent protein (kerateine). The kerateine derivatives differ in their isoelectric point and solubilities depending on the nature of the substituted group. In general, it may be stated that nearly all of the available –SH groups were substituted, while no detectable substitution of the amino groups occurred.

The antiserums to the keratin derivatives were prepared by immunization of rabbits with the substituted kerateines from feathers, while the derived proteins from wool kerateines were employed as test antigens. This was done in order to reduce the possibility of interactions between the parent proteins and their antiserums.

The precipitin reactions showed that the most marked precipitations occur at the interface of the proteins and their homologous antiserums.

The derived proteins containing the shorter aliphatic groups, *i. e.,* alpha carboxy-methyl kerateine and alpha carboxy-ethyl kerateine, gave the most specific reactions, while the range of activity broadens when the length of the side chain is increased in the deter-

3 Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.,* 1935, **18**, 308.

4 Goddard, D. R., and Schubert, M. P., *Biochem. J.,* 1935, **29**, 1009.

5 Michaelis, L., and Schubert, M. P., *J. Biol. Chem.,* 1934, **106**, 331.

minant groups. Some cross reactions were often encountered between compounds like alpha carboxy-amyl kerateine and alpha carboxy-n-butyl kerateine. A striking specificity was exhibited in the case of 2 compounds with benzene rings in their side chains. Benzyl kerateine was easily differentiated from compounds containing aliphatic side chains as "determinants."

These results compare favorably with those obtained by Landsteiner and van der Scheer[6] in their studies on antigens containing azo-components with aliphatic side chains.

With the exception of the azo-method introduced by Landsteiner the possibility of the introduction of determinant groups into proteins has been limited.

It has been generally assumed that the various methods employed affected the benzene ring, tyrosine being regarded as playing a major rôle in the determination of the specificity of proteins. The observations of Hopkins and Wormall,[7] in which the reaction of the free amino groups of proteins with phenyl-iso-cyanates was used to introduce new determinant groups, indicated that a protein may be altered immunologically by a process not affecting the benzene ring.

From the studies presented here it is therefore also evident that substitution of the –SH group of kerateines by simple chemical compounds influences the serological behavior as well as the chemical characteristics of these derived proteins.

10213 P

Influence of Balloon Distention of Duodeno-Jejunal Loops on Volume of Combined Digestive Secretions.*

M. Laurence Montgomery. (Introduced by Chauncey D. Leake.)

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In the course of certain studies which were designed to disclose whether acute intestinal obstruction is associated with the production of an increased flow of the digestive secretions, Swindt and

6 Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1934, **59**, 751.

7 Hopkins, S. J., and Wormall, A., *Biochem. J.*, 1933, **27**, 740.

* This work was conducted under grants from the Christine Breon Fund for Medical Research and the Melville Luther Montgomery Fund for Medical Research of the University of California.

Montgomery[1] observed that in isolated duodeno-jejunal loop strangulation obstruction the secretion of the combined digestive juices (gastric, pancreatic, biliary, and duodenal) is distinctly depressed. Careful analysis of the secretion curve showed that the depression occurred promptly and lasted for a period of from 12 to 24 hours, after which the secretion gradually returned toward normal and then decreased again as the animal became moribund. The early fall in secretion was often associated with nausea and vomiting and the appearance of mild symptoms of depression.

In addition we had observed in a single successful experiment of closed loop obstruction, performed on an animal with a denervated isolated loop after the "normal" fasting level of combined digestive secretions had been obtained, that no significant decrease in the secretion occurred until the animal was moribund, and that the animal showed no evidence of depression until shortly before death.

Since these findings were contrary to the general belief that acute strangulation obstruction produces a hypersecretion of the digestive juices[2] and since the alteration in secretion was associated with symptoms which Herrin and Meek[3] have attributed to nervous influences, we devised the following experiments to determine whether distention of a loop of bowel in the absence of the accumulation of "toxic" fluid within the loop would produce alterations in secretion similar to those observed in the experiments on closed, isolated loop strangulation.

Dogs were prepared with a 12- to 14-inch Thiry-Vella duodeno-jejunal fistula, the duodenum having been divided about 5-6 cm below the lower pancreatic duct. Dragstedt type cannulæ were then placed in the duodenum and jejunum proximally and distally, respectively, to the points of division of these structures for the purpose of collecting and replacing the combined digestive secretions. The loss of fluids by the kidneys and the lungs was made good by the daily intravenous or subcutaneous administration of from 1,000 to 1,500 cc of normal salt or Ringer's solution. In one series of 7 animals the nerves to the loop were not touched; in a second series of 4 animals the loop was denervated by severing all connections with the body except the blood supply. After normal fasting levels of secretion appeared to have been established a sausage-shaped balloon with a capacity of from 200 to 300 cc was

1 Swindt, Joseph M., and Montgomery, M. Laurence, PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 915.

2 Wangensteen, Owen H., *The Therapeutic Problem in Bowel Obstructions*, Springfield, Ill., C. C. Thomas, 1937, p. 6.

3 Herrin, Raymond C., and Meek, Walter J., *Arch. Int. Med.*, 1933, **51**, 152.

introduced into the distal end of the isolated loop and partially distended with 30 cc of water. Several hours later an additional 15 to 20 cc of water was placed in the balloon. These maneuvers were designed to simulate the trapping of fluid in obstructed loops. Then, in order to simulate a further gradual distention, water was introduced into the balloon at the rate of from 6 to 8 cc per hour, until the balloon contained a total of from 200 to 250 cc of water. The amount of distention used was based on the observation that the prerupture capacity of isolated closed loops of similar length and situation was about 200 cc.

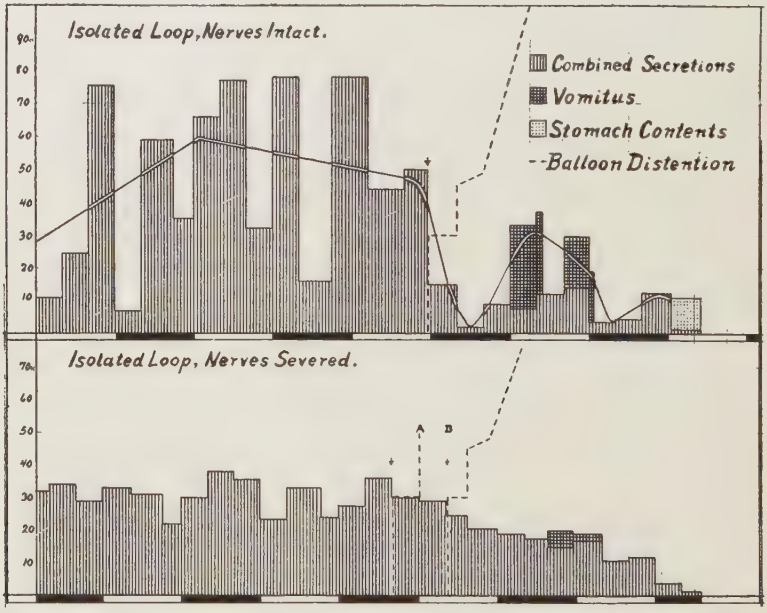

FIG. 1.
Secretion Curves.

Chart represents individual experiments in each classification, and is characteristic of the general trend.

The combined secretion is charted on the basis of cc per hour. The arrows show the points at which the balloon was introduced into the Thiry-Vella loop. In the experiment from which the lower graph was taken the balloon slipped out of the loop between points A and B and had to be replaced. The dotted lines show the rate at which the balloon was distended, using the same scale as that for the secretion. Solid lines represent noon to midnight. Non-solid lines midnight to noon.

Representative results are shown in Fig. 1. It will be observed that in the animal possessing an intact nerve supply to the loop of bowel mild distention of the balloon caused an early, marked suppression of the secretion similar to that observed in closed isolated

loop strangulation. In the animals in which the loop had been denervated the early suppression did not occur.

Conclusions. (1) Distention of an isolated loop of bowel to the point of strangulation, when caused either by the spontaneous accumulation of fluids therein, or by the distending of an indwelling balloon, produces an early temporary suppression of the combined digestive secretions. (2) The suppression of secretion does not occur when the loop has been denervated. (3) The similarity of the changes produced by balloon distention with those seen in spontaneous loop distention would seem to argue against the hypothesis that strangulation intestinal obstruction is associated with hypersecretion of the digestive juices.

10214

Nature of the Action of Testosterone on Genital Tract of the Immature Female Rat.*

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Opening of the vagina and estrus in the immature rat with the use of various androgens has been reported by Butenandt and Kudzus.[1] Deanesley and Parkes[2] noted, in addition, that testosterone and other androgens produced not only vaginal opening but also uterine enlargement in the ovariectomized as well as in the intact immature rat. This suggested, therefore, a direct action upon the uterus and vagina. Nelson and Merckel[3] described the uterine reaction of adult rats to androgens and found enlargement after the administration of testosterone, androstenedione, cis-androstenedione and dehydroandrosterone; the latter producing the response even in the absence of the pituitary. McKeown and Zuckerman[4]

* We are indebted to Dr. Joseph C. Aub for valuable suggestions in the preparation of the paper and to Dr. Shields Warren for reviewing the histological sections.

1 Butenandt, A., and Kudzus, H., *Hoppe-Seyler's Z.*, 1935, **237**, 75.

2 Deanesley, R., and Parkes, A. S., *Brit. Med. J.*, 1936, **1**, 527.

3 Nelson, W. O., and Merckel, C. G., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 825.

4 McKeown, T., and Zuckerman, S., *Proc. Roy. Soc. B.*, 1937, **124**, 362.

stated that the uterine response to testosterone differed in intact and ovariectomized animals. Brooksby[5] from other experiments concluded that the uterine response to testosterone differed qualitatively from that of progesterone and that of the estrogens. Recently, Salmon[6] with the use of testosterone propionate and androstenediol noted vaginal opening, uterine enlargement, and follicle stimulation with corpus luteum formation in the ovaries of the intact immature rat. He suggested that the vaginal opening may have resulted from a gonadotropic effect exerted upon the ovary, either directly or indirectly through stimulation of the hypophysis.

This study was undertaken to ascertain the mode of action of testosterone propionate† upon these various genital organs. All of the experiments listed below were carried out on immature white stock rats, weighing between 55 and 65 g. The animals mature generally at a weight of from 110 to 120 g.

Experiment 1. Ten intact immature female rats were given a single injection of testosterone propionate in sesame oil in doses varying from 2.5 to 10 mg. Five other animals were injected with equivalent amounts of sesame oil to serve as controls for this and subsequent experiments.

Results: (a) Vaginal opening occurred within 96 hours in all animals which received testosterone. The time of opening varied directly with the dosage employed. The controls showed no change. Vaginal smears within 12 hours after vaginal opening revealed complete or partial cornification in every instance (5 animals). Histologic sections of the vagina showed different stages of cornification (5 animals). This was not found in the control animals. (b) Ovaries removed within 24 hours after vaginal opening were enlarged and showed moderate to marked follicle stimulation (judged by mitotic activity and size of the follicle) in every instance (3 animals). In 2 cases, early luteal changes were seen. In ovaries examined 48 to 168 hours after vaginal opening more pronounced follicle stimulation was noted and corpora lutea in all stages were present in practically every ovary (7 animals). Here, too, the degree of change varied directly with the dosage employed. The ovaries from control animals showed no or only mild follicular activity. (c) The uteri in all animals were enlarged and exhibited moderate to marked edema. On microscopic section there was an increase in stroma, vascularity, and glands. Mitotic activity and

[5] Brooksby, J. B., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 235.

[6] Salmon, U. J., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 352.

† We are indebted to Doctors Gregory Stragnell and Erwin Schwenk of the Schering Corporation for a generous supply of testosterone propionate.

very slight secretory activity were noted in uteri which were removed 24 hours after vaginal opening (3 animals). In uteri removed when corpora lutea were present, there was marked secretory activity, with a marked increase in the number of glands (7 animals). The uteri of the control animals were small and showed no activity. (d) Tubes: When corpora lutea were present in the ovary, there was slight evidence of activity in the tubal epithelium, manifested by mitoses. This was not seen in any other group.

Experiment 2. Eight immature white rats were ovariectomized and 72 hours later were injected with testosterone as in experiment 1.

Results: (a) Vaginal opening and estrus (confirmed by smear and histologic examination of the vagina) occurred as in the intact animals. (b) The uteri removed at intervals of 24 to 168 hours after vaginal opening were enlarged and edematous. Microscopic examination showed an increase in stroma, vascularity and edema. There was also an increase in the number of glands, which, however, was not so marked as in the intact animal treated with testosterone. Secretory changes were not seen. (c) Tubes—no change.

Experiment 3. Eight immature animals were hypophysectomized by the parapharyngeal route, using a technic previously described.[7] Seventy-two hours later the animals were injected with testosterone as above. At the completion of the experiment the pituitary fossa of each of the animals was examined with a magnifying loupe to verify the fact that hypophysectomy had been complete.

Results: (a) Vaginal opening and estrus occurred as before. (b) The uteri resembled those of experiment 2. (c) The ovaries were small and showed only small, inactive follicles on histologic examination. (d) Tubes—no change.

Experiment 4. Five immature animals were hypophysectomized and, thereafter, at intervals varying from 72 to 240 hours were subjected to bilateral ovariectomy. Testosterone was administered approximately 72 hours after ovariectomy.

Results: The changes noted were the same as those in experiments 2 and 3.

From the results obtained it appears that the action of testosterone on the female genital tract of the immature rat is as follows:

1. A direct action upon the vagina, since the changes noted occurred in the absence of either or both the ovaries and hypophysis. Vaginal opening probably occurs as a result of an estrin-like action in the immature female rat. Warren[8] expresses the opinion that

[7] Franseen, C. C., Brues, A. M., and Richards, R. L., *Endocrinology*, 1938, **23**, 292.

[8] Warren, Shields, personal communication.

immature tissue is more labile and, therefore, may respond to a weaker estrous stimulus than adult tissue. This is based purely on speculation, although somewhat supported by other observations in vitamin deficiencies. Since other androgens have been shown to have moderate to marked estrogenic activity, it therefore remains a distinct possibility that testosterone, which belongs to the same series, may also have this same property, but to a much less degree, *i. e.,* it is not sufficient to evoke estrus in adult tissue in the doses commonly employed. It is possible, however, that testosterone is converted into an estrogen by the immature animal. Deanesley and Parkes[2] suggested that the ovary may be responsible for this change. Since the effect was also obtained in ovariectomized animals, this thesis seems unlikely. Changing of the chemical structure by the adrenal remains a possibility, but proof for this is lacking.

2. A direct action upon the uterus. In the absence of either the hypophysis or the ovary, changes in the uterus occur after testosterone is administered. This response, however, differs in the intact and ovariectomized immature animal. It seems significant that secretory changes were seen only in the intact animal and this when corpora lutea were present in the corresponding ovaries. The effect of testosterone on the uterus, therefore, in the intact and the ovariectomized animal seems to differ qualitatively as suggested by McKeown and Zuckerman[4] and Brooksby.[5] The secretory changes may occur as a result of the combined action of the ovarian hormones and testosterone; the ovarian hormones being elaborated as a consequence of the gonadotropic action of the hypophysis, which is stimulated by testosterone.

3. Changes in the tubes were noted only when corpora lutea were present in the ovary. This, therefore, can be attributed to an indirect action of testosterone.

4. An indirect action upon the ovary due to direct stimulation of the anterior hypophysis, which in turn elaborates the gonadotropic hormones to elicit follicle growth, maturation, and corpus luteum formation.

Conclusions. Testosterone stimulates directly the growth of the uterus and the vaginal epithelium of the immature rat. There is a qualitative difference only in the uterine response in the intact and ovariectomized animal. It does not act directly on the ovary but stimulates it indirectly by way of the anterior hypophysis, resulting in follicle maturation and corpus luteum formation. As a result of corpus luteum formation from hypophyseal stimulation, the Fallopian tubes may show growth activity.

10215

Inhibition of Experimental Dental Caries in the Rat by Fluoride and Iodoacetic Acid.

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The initial lesion of dental caries appears invariably in the outer enamel surface of the tooth. Since inflammation cannot occur in the completely mineralized, avascular enamel the carious process differs from most pathological lesions by the absence of any noticeable tissue reaction. Therefore the initial lesion may be considered as a localized decalcification—the result of an interaction between the chemical constituents of the tooth surface and the *outer* adjacent environment. Most observers believe that the initial lesion begins under a plaque of bacteria. If this be so, it appears plausible that these bacteria may themselves cause the "localized decalcification" (1) by dissolving out minerals by acids normally produced in carbohydrate metabolism and thus *incidentally* causing the carious area, or (2) by dissolving out phosphate, *of necessity,* to maintain their normal metabolic processes. Either process would depend on enzyme systems capable of transferring the phosphate radical. That the phosphorylation mechanism for carbohydrate breakdown described by Embden, Meyerhof and others in muscle and yeast also operates in bacterial metabolism has been indicated by Nelson and Werkman.[1]

If the above hypothesis is correct then it might be anticipated that specific inhibitors of the phosphorylating and dephosphorylating enzymes, such as fluoride and iodoacetic acid, should delay the initiation or spread of dental caries. To test this hypothesis, the effects of small amounts of fluoride and iodoacetic acid added to the food and water have been observed in rats placed on a diet capable of producing extensive caries in the molar teeth. These compounds are of particular interest because, in addition to their specific effect on the phosphorylating mechanisms involved in carbohydrate breakdown, they inhibit the enzyme system postulated for the calcification (and presumably decalcification) of bone.[2]

* The author wishes to thank Drs. R. W. Harrison, G. Gomori and J. R. Blayney for their kind cooperation.

1 Nelson, M. E., and Werkman, C. H., *J. Bact.*, 1936, **31**, 603.

2 Robison, R., and Rosenheim, A. H., *Biochem. J.*, 1934, **28**, 684.

Experimental. The caries-producing diet consisted of the following: 630 g of brown rice (Comet brand); 280 g dried milk powder; 30 g alfalfa meal; 50 g "Crisco" (vegetable fat); 10 g NaCl.

The Crisco was warmed just to the melting point. The rice was thoroughly stirred into the warm Crisco until all the grains were completely and uniformly coated; the rice was then mixed with the other constituents of the diet. When fluoride and iodoacetic acid were added to the diets these compounds were dissolved in the warm Crisco and coated on the rice as described above. By this method of preparation, each particle of rice received a permanent coating of the fluoride or iodoacetic acid. Since rats eat one grain of rice at a time, and do all their chewing with the molars, the method of preparation of the diets ensured adequate contact between the chemical compounds and the surfaces of the teeth during mastication. Control diets were prepared exactly as above except for substitution of 630 g of dried rolled oats for the rice.

The animals were divided as follows:

Group I. Twenty animals on the diet of rice and pure H_2O.

Group II. Twenty animals on the rice diet with fluoride added as follows: *Group II A.* 10 animals. Sodium fluoride, 250 mg per kilo of the complete diet. The fluoride was coated on the rice as described above. The water contained 4.2 mg NaF per liter. *Group II B.* 10 animals. Calcium fluoride, 500 mg per kilo of diet. Water containing 4.2 mg NaF per liter.

Group III. 10 animals. Iodoacetic acid, 200 mg per kilo of diet; 20 mg per liter of H_2O. The diet for this group also contained 4 g powdered baker's yeast per kg to obviate the toxic action of iodoacetic acid on intestinal absorption.[3]

Groups of 10 animals each were given the control diets containing the rolled oats.

Female, albino rats, all about 28 days old and of the same stock were placed on the various diets at the same time. They were fed continuously for 100 days at which time all the animals were sacrificed, and their lower jaws were divided at the symphysis. A count of the carious lesions on each side was made. In one count, made before completion of a simplified technic by Dr. George Gomori of the University of Chicago, one segment of the lower jaw was ground down in the sagittal plane and each of the 3 molar teeth examined. Numerous planes were examined under a dissecting microscope,

[3] Laszt, L., and Verzar, F., *Pflüger's Arch. f. d. Gesamte Physiol.*, 1938, **236**, 693.

TABLE I.

Group	Diet and H_2O	No. of animals	No. lower molar teeth examined	No. of lesions		
				Total	Per animal (X100)	Per tooth (X100)
I	Caries diet. Pure H_2O	17	102	30	177	30
II	" " plus NaF in food and H_2O	9	54	3	33	6
III	" " " CaF_2 in food, NaF in H_2O	10	60	9	90	15
IV	" " " iodoacetic acid in food and H_2O	10	60	1	10	2

TABLE II.

Group	Diet and H_2O	No. of animals	No. lower molar teeth examined	No. of lesions		
				Total	Per animal (X100)	Per tooth (X100)
I	Caries diet. Pure H_2O	17	51	16	96	31
II	" " plus NaF in food and H_2O	10	30	1	10	3
III	" " " CaF_2 in food, NaF in H_2O	10	30	3	30	10
IV	" " " iodoacetic acid in food and H_2O	10	30	0	0	0

magnification 30 ×, and definite areas of decalcification or actual cavitation were recorded. Later, the other half of the lower jaw was stained and cleared by the more exact Gomori technic (unpublished) and the 3 molars examined for carious areas. The upper molar teeth which are much less susceptible to experimental caries were not examined.

Results. The total numbers of lesions found by the 2 methods are grouped in Table I, and indicate that the animals fed sodium fluoride or iodoacetic acid, in addition to the caries-producing diet, show a very low incidence of caries as compared with the animals in Group I. The inhibiting-power of the calcium fluoride appears to be less marked than that of sodium fluoride or iodoacetic acid.

The results of the count performed by Dr. Gomori are recorded in Table II where the inhibitory effect of iodoacetic acid and sodium fluoride on the development of the rat caries is very strikingly demonstrated. This count represents one-half of the teeth recorded in Table I, but is probably equally significant because the improved histological method was employed. Both counts exhibit the same trends in each group, as shown by a comparison in Tables I and II.

The control animals on the oatmeal diet with or without addition of fluoride or iodoacetic acid did not develop any carious areas in the 100-day period.

The animals on each diet grew at a normal rate. Those fed sodium fluoride developed distinct mottling of the *incisor* teeth, and the ones given calcium fluoride slight mottling.

The results indicate a marked inhibition of experimental caries by both iodoacetic acid and sodium fluoride. Even though the numbers of animals studied are small it seems likely that the very great inhibition of caries caused by the iodoacetic acid and sodium fluoride represents significant results. The effect of calcium fluoride is less definite. (Calcium fluoride was tested in the hope that this insoluble fluoride would adhere to the teeth more tenaciously than sodium fluoride and act more effectively than the latter.)

The inhibitory compounds act probably by contact either (1) between the food and the flora of the tooth surface and/or (2) between the saliva and the tooth surfaces. The additional possibility that inhibiting quantities of iodoacetate or fluoride were incorporated into the tooth substance seems unlikely since the rat molars are rather completely developed and calcified by 28 days, the beginning of the experiment. Since knowledge of the action of fluoride and iodoacetic acid on protoplasm and cellular reproduction is still incom-

plete it is impossible to state definitely that the inhibitory effect of these compounds on dental caries results from specific effects on discrete enzyme systems, such as phosphorylation.

Summary. Sodium fluoride and iodoacteic acid added to a caries-producing diet fed to albino rats diminished greatly the incidence of carious lesions in the molar teeth.

10216

Phospholipids and Complementary Activity.

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In a previous communication,[1] it was demonstrated that prolonged extraction of active, dehydrated complement with organic solvents, *i. e.*, alcohol, ether, etc., resulted in no reduction of complementary activity. In fact, the recovered lipids often exhibited anticomplementary properties when returned to the extracted residues prior to titrations.

Recently, Bloor and Snyder[2] have noted that a buffered suspension of oxidized phospholipids oxidizes a sensitive methylene blue preparation.

In view of earlier work from this laboratory,[3] which revealed an intimate connection between complementary powers and oxido-reduction phenomena, it was decided worthwhile to study the effect of oxidized and unoxidized cephalin and lecithin on complementary activity.

Purified preparations of the 2 phospholipids were employed throughout these experiments. The oxidized phospholipids were prepared according to the technic of Bloor and Snider and complement was titrated by the method advocated by Ecker, Pillemer, Wertheimer and Gradis.[4]

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* Crile Fellow in Pathology.

1 Ecker, E. E., Pillemer, L., and Grabill, F. J., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 318.

2 Bloor, W. R., and Snider, R. H., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 215.

3 Ecker, E. E., Pillemer, L., Martiensen, E. W., and Wertheimer, D., *J. Biol. Chem.*, 1938, **123**, 351.

4 Ecker, E. E., Pillemer, L., Wertheimer, D., and Gradis, H., *J. Immunol.*, 1938, **34**, 19.

The amounts of reagents employed and general procedures are found in the accompanying protocols. All solutions were prepared in a M/15 phosphate buffer pH 7.2.

The experiments of Bloor and Snider were repeated and confirmed. As reported by these authors, it was also found that phospholipids do not oxidize a sensitive preparation of reduced methylene blue, while the oxidized lipids show a definite oxidizing power.

TABLE I.
Showing the Relative Inhibiting Actions of Various Phospholipids on Complementary Activity.

Phospholipids	Concentrations of the phospholipids necessary to inactivate 1 cc of a 1:10 dilution of complement
	%
Cephalin	.008
Oxidized cephalin	.05
Lecithin	.10
Oxidized lecithin	.40

Various portions of the phospholipids were then added to fresh guinea pig complement, and their effects noted. Table I shows the relative inhibitory effect of the various phospholipids on complement. Their inhibitory powers were found in the following orders:

Unoxidized cephalin > oxidized cephalin > unoxidized lecithin > lecithin

It is of interest, and likewise difficult to explain, that the unoxidized preparations were more inhibitory than the oxidized preparations.

The effect of ascorbic acid and of reduced glutathione were then studied with regard to complements inactivated by these phospholipids. These results are found in Table II.

From the data obtained, it is evident that ascorbic acid and glutathione SH possess the ability to restore the diminished complementary activity of the complements after treatment with lipids. The most marked reactivation was accomplished with serums inactivated by unoxidized cephalin, while the others proceeded in the following order:

Unoxidized lecithin > oxidized cephalin > oxidized lecithin. In fact, only a slight reactivation was accomplished with the complements treated with the oxidized lecithin. Another point of interest is the observation that ascorbic acid yielded the most marked and consistent reactivations.

Similar findings were obtained when the reductants were incubated first with the phospholipids and then complement added to

TABLE II.
Reactivation of Complement Inhibited by Various Phospholipids.

Complement 1:10	Initial Hemolysis									
	.01 cc	.02 cc	.03 cc	.04 cc	.05 cc	.06 cc	.07 cc	.08 cc	.09 cc	.10 cc
+ 2 cc M/15 buffer pH 7.2*	O	H	H	H	H	H	H	H	H	H
" 1 " cephalin 1:24,000 + 1.0 cc buffer	O	O	O	O	O	O	O	O	O	H
" " " " " " " .5% ascorbic acid	O	O	H	H	H	H	H	H	H	H
" " " " " " " .5% GSH	O	O	O	O	O	H	H	H	H	H
" " " oxid. cephalin 1:2,000 + 1.0 cc buffer	O	O	O	O	O	O	O	O	H	H
" " " " " " " " .5% ascorbic acid	O	O	O	O	O	H	H	H	H	H
" " " " " " " " .5% GSH	O	O	O	O	O	O	H	H	H	H
" " " lecithin 1:2,000 + 1.0 cc buffer	O	O	O	O	O	O	H	H	H	H
" " " " " " " .5% ascorbic acid	O	O	H	H	H	H	H	H	H	H
" " " " " " " .5% GSH	O	O	O	O	H	H	H	H	H	H
" " " oxid. lecithin 1:500 + 1.0 cc buffer	O	O	O	O	O	O	H	H	H	H
" " " " " " " .5% ascorbic acid	O	O	O	O	O	H	H	H	H	H
" " " " " " " .5% GSH	O	O	O	O	O	O	H	H	H	H

H = Hemolysis.
* = Phosphate buffer.

The phospholipids were incubated for 1 hour with the serums, and then the inactivated complements were incubated for 30 minutes with the various reductants. Ascorbic acid and GSH were prepared in buffer solution 7.2 M/.15.

the mixture. This would indicate that the protective phenomenon observed was due to the interaction of the reductants with the phospholipids, thereby inhibiting their anticomplementary powers.

It has been generally believed that phospholipids play a distinct rôle not only in biological redox phenomena[5, 6] but also in immunological reactions. It is therefore possible that a connection exists between these two assumptions.

Evidence presented here indicates that an excess of cephalin or of lecithin weakens complement and that the effect may be reversed by ascorbic acid or by glutathione SH. These findings, therefore, point to the fact that the phospholipid inactivation of complement may be at least partly oxidative in nature.

The reactivations of the inactivated complements by ascorbic acid and by reduced glutathione may be due either to the reduction capacity of these agents, thereby reducing the oxidized system to a state whereby it can function or that the reductants employed unite directly with the phospholipids and thereby prevent the compounds from exerting an anticomplementary action.

It is at present difficult to explain the differences observed between the oxidized and the unoxidized phospholipids.

10217

Effect of Divinyl Oxide on Intestinal Activity *in vivo*.*

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The knowledge of the effect of the newer inhalation anesthetic agents on intestinal contraction is far from adequate. In a previous report[1] it was indicated that during the first two planes of surgical anesthesia the effect of cyclopropane on intestinal activity in the intact animal consists of an increase of both intestinal contractions and tone followed by inhibition if narcosis is further deepened. These results agree with the *in vitro* effects observed by Peoples and

[5] Koch, W., *Ztschr. Physiol. Chem.*, 1903, **37**, 181.

[6] Fränkel, S., and Dimitz, L., *Wien. klin. Wchnschr.*, 1909, **22**, 1777.

*Grateful acknowledgment is expressed to Dr. W. F. Ruggiero for his aid in the surgical preparation of the Thiry-Vella Fistulas.

[1] Burstein, C. L., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 530.

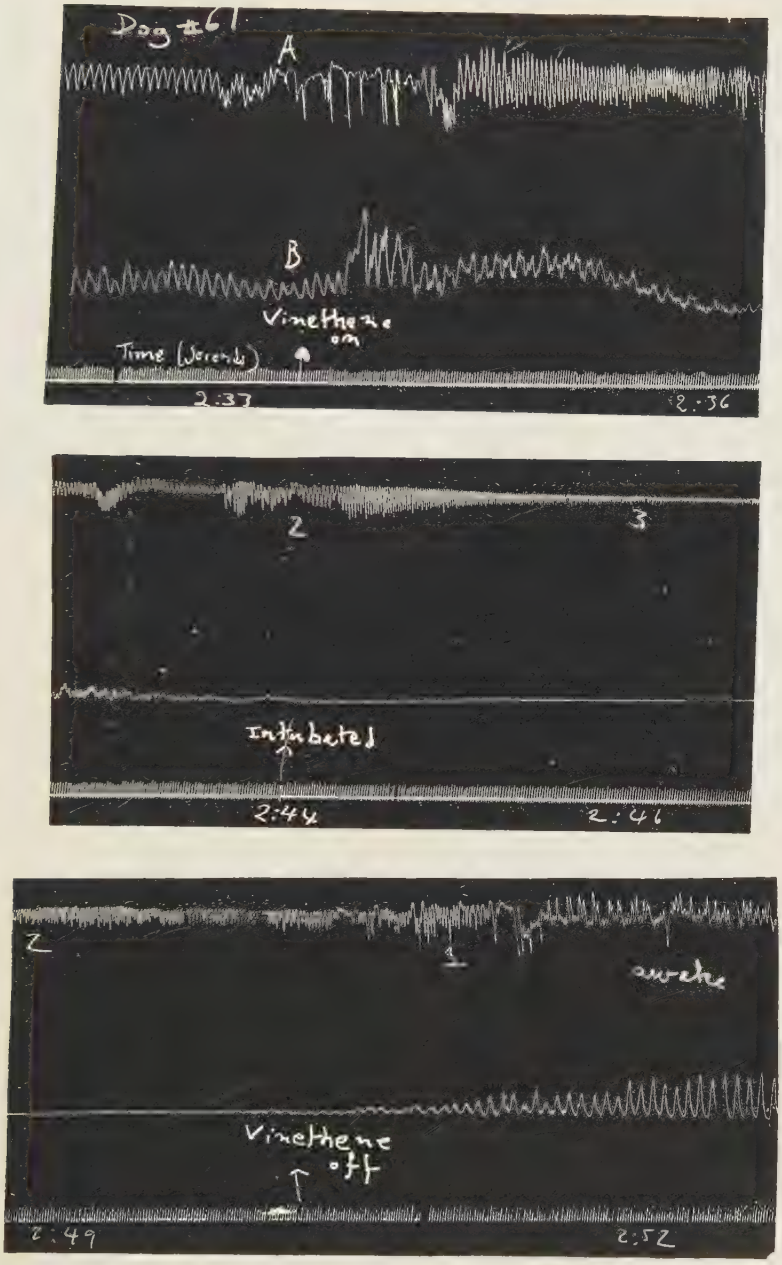

FIG. 1.

Effect of divinyl oxide on intestinal activity of dog *in vivo*. Serial sections taken from the same experiment: Upper record (A), thoracic respiration; lower (B), intestinal contractions. Time in second intervals. The numbers below the respiratory tracing indicate the plane of surgical anesthesia. Divinyl oxide (Vinethene) was administered between arrows (2:34 to 2:50).

Phatak[2] on isolated intestinal muscle. Divinyl oxide, another of the newer anesthetic agents, was shown by the same authors[3] likewise to increase the tonicity of the isolated intestinal segment. The present study is concerned with the effect of divinyl oxide on intestinal activity *in vivo*.

Six dogs were prepared with Thiry-Vella loops of the upper jejunum. When healing had occurred, tracings of the intestinal movements were recorded after inserting a balloon, connected to a water manometer, into the lumen of the proximal end of the Thiry-Vella loop. Respiratory tracings by means of a pneumograph applied about the chest were simultaneously recorded in order to rule out the possibility that the tracings taken from the balloon were not due to respiratory movements. No preanesthetic medication was administered. All animals were anesthetized with divinyl oxide (Vinethene—Merck) and oxygen by means of a Foregger Metric machine using the closed carbon dioxide-absorption technic.[4] An endotracheal tube fitted with an inflatable cuff was introduced as soon as the state of surgical anesthesia was reached in order to assure a patent airway and thereby obviate the effect of respiratory obstruction and the ensuing anoxemia.

Results. Contrary to the *in vitro* results, all animals showed effects identical to those obtained with ether (di-ethyl oxide); namely, diminished muscular tone and complete inhibition of intestinal contractions during all planes of surgical anesthesia. (Fig. 1.)

10218

A Fraction from Normal Chick Embryo Similar to the Tumor Producing Fraction of Chicken Tumor I.

ALBERT CLAUDE.

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A fraction possessing high tumor-producing activity can be isolated from chicken sarcoma extracts by means of differential cen-

[2] Peoples, S. A., and Phatak, N. M., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 287.

[3] Peoples, S. A., and Phatak, N. M., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 378.

[4] Rovenstine, E. A., *Am. J. Surg.*, 1936, **34**, 456.

trifugation at high speed.[1] Application of the same technic to the fractionation of normal chick embryo extracts has resulted in the separation of a fraction presenting similar chemical and physical properties but exhibiting no tumor-producing power.

Eight-day-old chick embryos were used in these experiments. The whole embryo was washed several times in sterile saline solution and then forced through a *Latapie* masher. The resulting watery pulp was frozen at –80°C and stored at that temperature from 3 to 30 days.

The embryo extract was prepared by grinding the frozen tissue with sand and extracting with a phosphate buffer solution.* The suspension was centrifuged at 2400 times gravity for 15 minutes and the deposited tissue was reëxtracted in the same manner. The sediment was discarded and the supernatant fluids from the two extractions were combined and filtered through sterile gauze. The total volume of buffer used for this double extraction corresponded to 15 times the weight of the fresh embryo pulp.

Embryo extracts prepared in this way are opalescent solutions containing about 3.2 mg solids per cc† and having a specific viscosity of about 1.2 as measured in the Ostwald viscosimeter.

Fractionation of this embryo extract by high speed centrifugation was accomplished as follows: The extract was first centrifuged one hour at 17,000 r.p.m. At that speed the corresponding centrifugal force, in the center of the tube, is about 18,000 times that of gravity.‡ The supernatant liquid from this long run at high speed was discarded and the jelly-like sediment was taken up in phosphate buffer solution. This suspension was submitted to a short run of 3 minutes at high speed and the supernatant fluid was saved. The deposit was taken up once more in a small volume of buffer solution and the suspension centrifuged for 3 minutes at high speed. The deposit was discarded and the supernatant fluid combined with that of the previous run. This solution was used for the next series of centrifugation. The whole process consisting in the elimination of the soluble elements of the extract by a long run of one hour at

[1] Claude, A., *Science*, 1938, **87**, 467.

* The extraction, and subsequent washings in the centrifuge, were made with a 0.005M phosphate buffer solution at pH 7.0.

† This figure represents the extracted solids, deduction being made for the salt content from the buffer solution.

‡ The centrifuge used in this work was the Type B, size 1 model, with the multispeed attachment and No. 295 head, of the International Equipment Co., Boston, Mass.

high speed, followed by the removal of the coarse particles by two short runs of 3 minutes, was repeated twice.

During the entire experiment the temperature of the material was maintained near 0°C, except during the long runs at high speed when it attained 12 to 14°C.

The purified fraction isolated by this method forms opalescent solutions in phosphate buffers at pH 7.0. In the dark field microscope§ the material appears to be composed of minute granules similar to those found, under the same conditions, in purified and active chicken tumor preparations. When deposited in the high speed centrifuge, the purified chick embryo fraction appears as a pale yellow or nearly colorless, perfectly transparent, mass. Dried *in vacuo* in the frozen state, the substance is a snow white material which will yield 5% more moisture when heated at 80°C.

Under the above conditions of preparation the fraction separated by high speed centrifugation corresponds, in terms of dry weight, to 5.4% of the original embryo extract, or about 2.9 mg dried substance per gram of fresh embryo pulp.

The behavior of the embryo fraction in acid buffer solutions presents a striking similarity to that of the active fraction isolated by the same technic, from chicken tumor extracts,[1] and the solubility curves obtained in each case are practically identical. Solutions containing 0.17 mg of embryo fraction per cc are completely precipitated between pH 2.2 and 5.0. Beyond these two points, the substance becomes more soluble, and at pH 1.0 and 6.2 respectively, the acid mixtures present the normal opalescence of the neutral solution. The point of maximum precipitation, and probably the isoelectric point, of the material is near pH 3.6.||

Chemical analysis was performed on the purified fraction washed 3 times in cold distilled water, desiccated *in vacuo* in the frozen state, and dried further by keeping the residue 6 hours at 80°C. The results indicate that about 8.5% of the material is nitrogen and 1.8% is phosphorus. About 47% of the dried substance was found to be soluble in ether. The lipoid material can be fractionated into 2 main components, by means of their different solubilities in 80% alcohol. As in the case of the purified tumor fraction the major part of the lipoids from the embryo material presents the properties of lecithin. The less abundant fraction which is soluble

§ We are indebted to Dr. P. Olitsky for the use of the dark field microscope and carbon arc lamp.

|| The hydrogen ion concentration of the mixtures was determined by means of a glass electrode potentiometer.

in chloroform and pyridine, but which is insoluble in other ordinary organic solvents, may belong to the group of cerebrosides.

The portion of the purified fraction which is not soluble in ether contains 16.5% nitrogen and gives positive tests for proteins. The Feulgen test for thymonucleic acid is strongly positive.¶

The absorption spectrum of a freshly prepared solution of the embryo fraction was determined by Dr. A. Rothen. A 0.024% solution of the material in 0.005 M phosphate buffer at pH 7.0 gives a maximum absorption in the wave length region of 2600 Å and the curve is very similar if not identical with that given by certain solutions, at the same concentration, of the purified fraction obtained from active chicken tumor extracts.[2] These results are shown in text Fig. 1.

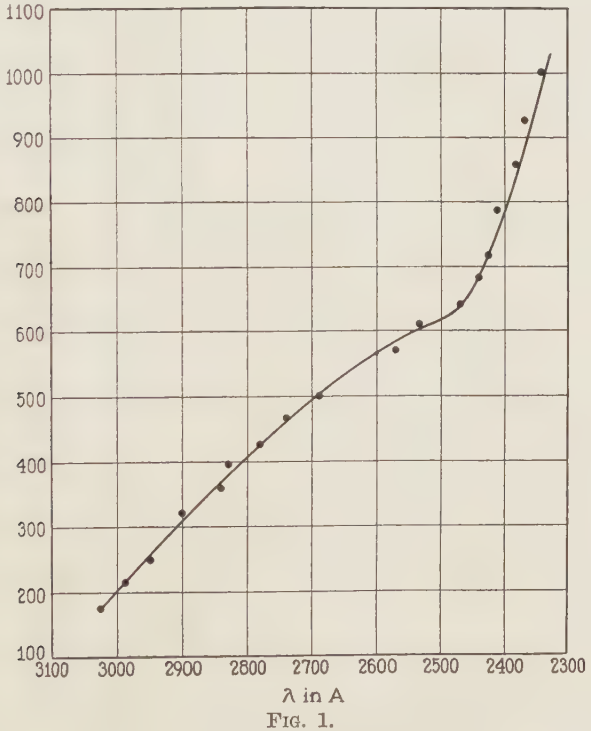

FIG. 1.

Ultraviolet absorption spectrum of the purified fraction prepared from chick embryo tissue by high speed centrifugation. 0.024% solution in 0.005 M. Phosphate buffer, pH 7.0.

¶ Concentrated, purified preparations from chicken tumor I give also a positive test for thymonucleic acid.

2 Claude, A., and Rothen, A., *Am. J. Cancer*, 1936, **26**, 344; Claude, A., *Am. J. Cancer*, 1937, **30**, 742; Claude, A., and Rothen, A., in preparation.

The purified fraction from chick embryo was tested for possible tumor-producing activity by injecting a freshly prepared solution, at various concentrations, into the skin of normal Plymouth Rock hens. The tests have been completely negative, as far as the production of tumors is concerned.

It has been shown that the principle causing a chicken sarcoma can be concentrated in a high speed centrifuge[3] and more recently this method has been utilized to isolate and purify the active material for chemical and physical examination.[1] On dark field microscopic examination this fraction appears to be composed of fine granules, the size of which has been estimated at about 70 mμ in diameter,[4] with a corresponding weight of 2.34×10^{-16} g, as calculated for a density of 1.3.[5] Several workers are inclined to consider these granules as representing the actual tumor agent.[6]

In the present investigation it is demonstrated that chick embryo tissue extracted and centrifuged in the same manner will yield a fraction which, in its main physical and chemical characteristics, is very similar to the active material from the chicken tumor but without tumor-producing properties. The estimated size of the particles are practically the same and the dark field microscopic examination shows the deposit to be made up of the same type of granules.

The purified fraction represents 3.5% of the original tumor extract against 5.4% for the chick embryo extract. Both substances, either from chick embryo or from chicken tumor, have the same solubility in acid solutions, they absorb ultraviolet light in the same manner and both are made up of lipoids and proteins. Color tests and absorption spectrum analysis indicate that one of the main compounds is a nucleoprotein. However, it will be noted that the ether-insoluble portion of the chicken tumor has a nitrogen content of 13.11% while the corresponding fraction of the embryo has 16.5%. It is possible that this difference may be significant. These findings raise the question whether the main constituents of the purified chicken tumor fraction represent inert elements existing also in normal cells or whether the substance found in normal chick embryo tissue may represent a precursor of the chicken tumor principle which could assume, under certain conditions, the self-perpetuating properties of the tumor agent.

[3] Ledingham, J. C. G., and Gye, W. E., *Lancet*, 1935, **1**, 376; McIntosh, J., *J. Path. and Bact.*, 1935, **41**, 215.

[4] Elford, W. J., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1936, **17**, 422.

[5] Claude, A., *J. Exp. Med.*, 1937, **66**, 59.

[6] Ledingham, J. C., and Gye, W. E., *Lancet*, 1935, **1**, 376; Amies, C. R., *J. Path. and Bact.*, 1937, **44**, 141.

These observations emphasize the necessity of ascertaining further the rôle played by the two chief components of the tumor fraction, namely the lipoids and the nucleoproteins, in the production of tumors.

Summary. By means of a method of differential centrifugation at high speed, a fraction can be separated from normal chick embryo tissue, which, in its main characteristics, resembles the active fraction isolated from chicken tumor extracts by the same method.

The implications of these observations are discussed.

10219

Constancy of Urea Clearances in Dogs Following Surgical Anesthesias with Cyclopropane, Ether, and Chloroform.*

O. S. ORTH AND J. W. STUTZMAN. (Introduced by W. J. Meek.)

From the Physiology Department, University of Wisconsin Medical School, Madison.

Perusal of the literature reveals no extended or correlated study of the effects of the common anesthetics on normal kidney function. Most of the work has been limited to determination of the rate of urine secretion before and after the various anesthetics.[1-4] Studies by Haines, *et al.*,[5] on the effects of ether, morphine, and atropine in various combinations upon dye excretion (phenol red and indigo carmine) before and *during* the period of anesthesia, and of Stehle and Bourne[6] on urine flow, urea, and chloride during and for 3 hours after ether, morphine, or both, have been along similar lines, although with conflicting results. Studies of urea, chloride, phosphate, and water excretion under ether, ethylene, ethylene and amytal, and ethylene and tribromethanol have been carried out by Walton[7] who found no significant changes. Recently Greisheimer, *et al.*,[8] in studies on one dog reported urea clearance elevations of 30%

* Aided in part by a grant from the Wisconsin Alumni Research Foundation.

1 Bonsmann, M. R., *Arch. f. exp. Path. u. Pharm.*, 1930, **156**, 160.

2 Buxton, D., and Levy, A. G., *Brit. M. J.*, 1900, **2**, 833.

3 Kemp, R. C., *New York M. J.*, 1899, **70**, 732.

4 Thompson, W. H., *Brit. M. J.*, 1906, 608 and 667.

5 Haines, W. H., and Milliken, L. F., *J. Urol.*, 1927, **17**, 147.

6 Stehle, R. L., and Bourne, W., *Arch. Int. Med.*, 1928, **42**, 248.

7 Walton, R. P., *J. Pharm. and Exp. Therap.*, 1933, **47**, 141.

8 Greisheimer, E. M., Hafkesbring, R., and Magalhaes, H., *Am. J. Physiol.*, 1938, **123**, 85.

on the days following cyclopropane anesthetization, and a return to 7% above normal within 5 to 7 days, with the control level being reached in 3 to 4 weeks.

In the course of other studies being made on cyclopropane anesthesia it was deemed advisable to determine the effect of this agent on kidney function as measured by urea clearance. The effects of ether and chloroform also were determined.

Urea clearance determinations have been made on a group of 5 adult female dogs kept in stock kennels and fed a standard diet of dog biscuits throughout the entire 8 months' test period. The animals have been in our kennels for periods of 10 months to more than 2 years and are all in a very healthy condition. A 4 to 6 weeks' adjustment period to the animal room environment was allowed before any animal was used.

The animals were trained to lie quietly on their backs and submit to catheterization and arterial puncture. The technic of Van Slyke[9] was followed in making the urea clearance determinations, all of which were post-absorptive, maximal, and expressed in cc per meter squared per minute, surface area being determined by the formula of Cowgill and Drabkin.[10] Three or more successive periods of 20 to 30 minute urine collections were made, urine samples of less than 0.4 cc per minute being discarded. The hypobromite method of urea analysis[11] was used routinely, since it simplified the procedures and is considered by Van Slyke[12] to be accurate. Urine simples were tested chemically for albumin and glucose and examined microscopically.

The general plan was first to determine the normal clearance values and then to subject each animal to surgical anesthesia for a period of 60 to 75 minutes. Following this the animals were placed in metabolism cages for checking the return of urine flow to normal and on succeeding days clearance determinations were repeated. Two of the dogs, which had not been subjected to *any* previous anesthetization, were given 3 periods of cyclopropane anesthesia at weekly intervals and a fourth period at the end of 14 weeks. After a rest of 2 months these same dogs were each given 2 chloroform anesthetizations 6 days apart. The other 3 animals were submitted to 2 or 3 etherizations at weekly intervals. These dogs had previously received cyclopropane in the course of other studies, but at least one month had intervened.

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9 Van Slyke, D. D., Hiller, A., and Miller, B. F., *Am. J. Physiol.*, 1935, **113**, 611.

10 Cowgill, G. R., and Drabkin, D. L., *Am. J. Physiol.*, 1927, **81**, 36.

11 Van Slyke, D. D., and Kugel, V., *J. Biol. Chem.*, 1933, **102**, 489.

12 Van Slyke, D. D., *Am. J. Med. Tech.*, 1936, **2**, 42.

TABLE I.

Summary of urea clearances before (control) and after cyclopropane, ether, or chloroform anesthetization. All clearances were maximal and are calculated in cc per square meter per minute. All anesthetizations were at deep surgical level and each period of anesthesia was for an hour or more.

Dog	Anesthetic Agent	No. of Anesthetizations	No. of Clearance Determinations Before Anesthesia	No. of Clearance Determinations After Anesthesia	Maximal Clearances Averages in cc/M²/min. Before Anesthesia	Maximal Clearances Averages in cc/M²/min. After Anesthesia	Coefficient of Variation Before Anesthesia	Coefficient of Variation After Anesthesia
A	Cyclopropane	4	26	14	34.7 ± 6.1	33.0 ± 5.2	17.5	15.7
B	”	4	22	13	32.6 ± 6.7	35.5 ± 5.6	20.6	15.8
	Totals	8	48	27	Avg 33.6	34.2		
C	Ether	3	21	15	34.9 ± 6.1	38.8 ± 4.5	17.5	11.6
D	”	2	5	12	32.7 ± 2.4	27.4 ± 2.2	7.3	8.0
E	”	2	6	12	42.5 ± 3.8	41.3 ± 2.9	8.9	7.0
	Totals	7	32	39	Avg 36.7	35.8		
A	Chloroform	2	5	9	39.7 ± 3.1	34.9 ± 2.3	8.0	6.6
B	”	2	6	8	34.0 ± 3.2	34.5 ± 4.1	9.4	11.9
	Totals	4	11	17	Avg 36.8	34.7		
	Grand totals	19	91	83	Composite avg 34.9 ± 6.1	35.2 ± 5.8	17.6	16.6

Constancy of chloroform and cyclopropane anesthetization was carried out as previously described.[13] Constant depth of ether anesthesia was maintained by connection with an endotracheal tube to an ether bottle, a soda-lime carbon dioxide absorber being interposed. In all the experiments surgical anesthesia was considered present when the eye reflexes were absent and at least partial intercostal paralysis occurred. In most of the anesthesias complete intercostal paralysis prevailed.

As reference to Table I will show, the average of 27 clearances following the 4 periods of cyclopropane anesthesia to each of 2 animals is 34.2 cc/M²/min. Forty-eight control clearances on the same animals averaged 33.6 cc/M²/min. For dog A the clearances were 34.7 ± 6.1 before and 33.0 ± 5.2 after cyclopropane anesthesia; for dog B the corresponding values were 32.6 ± 6.7 and 33.5 ± 5.6 cc/M²/min. The coefficients of variation for A were 17.5 and 15.7 and for B 20.6 and 15.8. In Fig. 1 are plotted all the

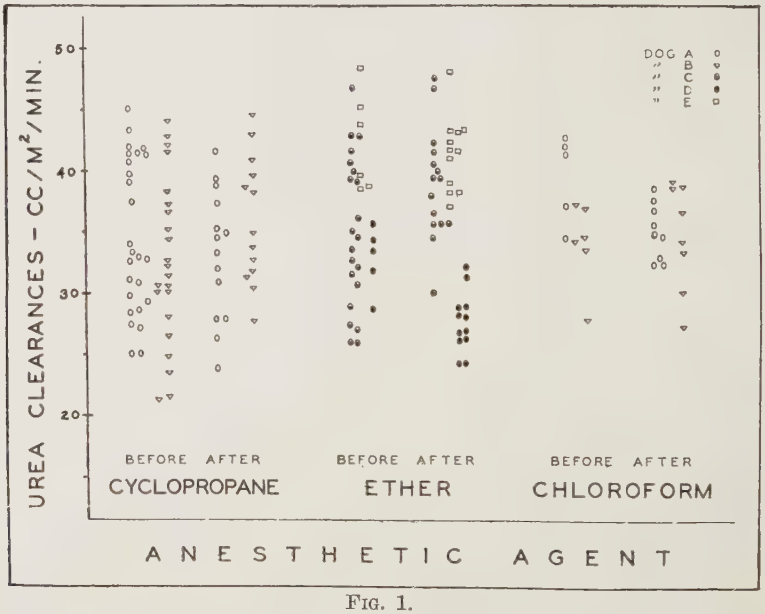

FIG. 1.

Maximal urea clearances in cc/M²/minute on 5 dogs as determined before (control) and after anesthetization with various agents. Dogs A and B 4 periods each with cyclopropane, and 2 months after the last treatment with this agent 2 periods each with chloroform. Dog C, 3 etherizations and dogs D and E, 2 etherizations each. All anesthetizations were at deep surgical level, and each period of anesthesia was for an hour or more.

[13] Meek, W. J., Hathaway, H. R., and Orth, O. S., *J. Pharm. and Exp. Therap.*, 1937, **61**, 240.

data for the 2 animals, grouped by clearances before and after anesthetization.

Thirty-two control determinations for the 3 animals studied with ether have a mean value of 36.7 cc/M²/min, and 39 clearance determinations after 7 anesthetizations average 35.8 cc/M²/min. The clearances of the 3 animals before etherization were 34.9 ± 6.1, 32.7 ± 2.4, and 42.5 ± 3.8 respectively, the coefficients of variation being 17.5, 7.3, and 8.9. After the anesthetizations the corresponding values were 38.8 ± 4.5, 27.4 ± 2.2, and 41.3 ± 2.9, with coefficients of variation of 11.6, 8.0, and 7.0. These data are plotted in Fig. 1 and summarized in Table I.

Seventeen clearances following the 4 periods of chloroform anesthetization of the same animals used in the cyclopropane study average 34.7 cc/M²/min. The 11 control clearances averaged 36.8 cc/M²/min. For dog A the clearances were 39.7 ± 3.1 before and 34.9 ± 2.3 after chloroform anesthesia, with corresponding coefficients of variation of 8.0 and 6.6. For dog B the corresponding values were 34.0 ± 3.2 and 34.5 ± 4.1 with coefficients of variation of 9.4 and 11.9. Fig. 1 and Table I include these data.

It will be noted that for the 5 animals the average of 91 control clearances was 34.9 ± 6.1 cc/M²/min, with a coefficient of variation of 17.6. For the 83 determinations following a total of 19 anesthesias, given at 10 different times, with cyclopropane, ether, and chloroform, clearances average 35.2 ± 5.8 cc/M²/min, with a coefficient of variation of 16.6.

Repeated collections of 24-hour urine samples following each of the anesthetic agents have shown normal volumes within this period of time. No significant alteration was found upon microscopic examination of the urine, although slight casts, leucocytes, and occasional erythrocytes were present. After ether anesthesia a slight albuminuria was present but cleared within a few days. Cyclopropane and chloroform did not elicit these results. Glucosuria was never present.

The results of these tests seem to indicate that none of the 3 anesthetic agents studied—cyclopropane, ether, or chloroform—interferes with kidney function, as determined by urea clearance. Since urea is the chief product of excretion in the urine and involves activity of both the glomeruli and tubules in filtration and reabsorption it is felt that such determinations give satisfactory indices of the effect of these anesthetic agents on the kidney. While slight fluctuations in clearance values occur, they merely represent the normal variation in function of the kidney, as recently emphasized by Van Slyke.[12]

It is to be noted from Table I that the animals were subjected to much more exposure to any one of the agents than usually occurs clinically. In addition dogs C, D, and E had received 4, 9, and 7 cyclopropane anesthesias in the year previous to the studies under ether, yet their control clearances were in the same range as those of dogs A and B which had been submitted to *no* previous experimentation. This is further proof, though indirect, of the innocuousness of cyclopropane on the kidney.

Direct proof is afforded by dogs A and B (Table I) in which control clearances average 33.6 and clearances after cyclopropane 34.2 cc/M^2/min, a surprisingly close agreement considering the normal functional fluctuations which have been shown to occur in this vital organ. Likewise in Table I is shown strikingly the normal functioning of the kidneys following ether and chloroform anesthetizations, clearances before anesthetization being practically identical with those after either of the agents. Since the same 2 animals that had been tested with cyclopropane showed no kidney injury under chloroform, one feels all the more certain that these agents do not interfere with kidney function, at least as tested by the method of urea clearance.

Summary. On a group of 5 adult female dogs maximal urea clearance determinations following anesthetization with cyclopropane, ether, or chloroform have shown no significant variations over a period of 8 months from control values. Ninety-one control determinations made on the 5 animals before anesthetization gave average clearance values of 34.9 ± 6.1 cc per square meter per minute with a coefficient of variation of 17.6 and 83 determinations made after 19 anesthesias with the three agents averaged 35.2 ± 5.9 cc per square meter per minute with a coefficient of variation of 16.6.†

† We wish to thank Prof. R. C. Herrin for continued advice during the course of this problem, and Dr. M. Digby Leigh of the Anesthesia Department for helpful assistance.

10220

Effect of Adrenal Cortical Extract on Experimental Hyperthyroidism in Dogs.

R. J. Schachter. (Introduced by A. J. Carlson.)
From the Department of Physiology, University of Chicago.

Marine, Lowe, and Cipra[1] suggested that the increased heat production of newly-born infants during their second week of life was due to the involution of the adrenal cortex, Marine and Baumann[2] reported that the O_2 consumption of rabbits is increased, when the adrenal cortex is destroyed either in part or *in toto*, and the increase in O_2 consumption is proportional to the amount of adrenal cortical tissue destroyed. Shapiro and Marine[3] reported that the symptoms of hyperthyroidism were alleviated in a patient with Graves' disease, upon the eating of raw desiccated adrenal tissue. From all the above data Marine[4] concludes that decrease in adrenal cortical secretion may be a factor in causing hyperthyroidism.

Oehme[5, 6] reports that injection of cortin into guinea pigs, prevents the usual action of thyroxin, while Elmer, Giedosz, and Schepps[7] report that in guinea pigs cortin will not prevent the increased O_2 consumption due to the thyrotropic hormone.

The work was done on 3 adult dogs, 2 males and one female. The dogs, for a period of 2 months, before the basal metabolic rate (B.M.R.) determinations were started, were kept on a standard diet of meat, bread, and bonemeal.

Daily B.M.R. were determined for 2-week periods under the following conditions.

1. Dogs on normal diet.
2. Dogs on normal diet plus adrenal cortical extract.
3. Dogs on normal diet plus 0.4 g of desiccated thyroid per kg of body weight.
4. Dogs on normal diet plus 0.4 g of thyroid per kg of body weight plus adrenal cortical extract.
5. Dogs on normal diet plus 0.4 g of thyroid per kg of body weight.

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1 Marine, D., Lowe, B. H., and Cipra, A., *J. Metab. Research*, 1922, **2**, 329.
2 Marine, D., and Baumann, E. J., *Am. J. Physiol.*, 1921, **57**, 135.
3 Shapiro, S., and Marine, D., *Endocrinol.*, 1921, **5**, 699.
4 Marine, D., *Am. J. Med. Sc.*, 1930, **180**, 767.
5 Oehme, C., *Klin. Wochenschr.*, 1936, **15**, 512.
6 Oehme, C., *Path. u. Pharmak.*, 1937, **184**, 558.
7 Elmer, A. W., Giedosz, B., and Schepps, M., *C. R. Soc. Biol.*, 1935, **118**, 1373.

TABLE I.

Dog	L. O_2 consumed per kg per 24 hr			% increase above normal	L. O_2 consumed per kg per 24 hr thyroid with adrenal extract	% increase above normal	L. O_2 consumed per kg per 24 hr thyroid	% increase above normal
	Normal	Adrenal extract	Thyroid					
W	9.2	9.1	11.7	27	13.8	50	13.3	45
B	11.5	11.2	13.8	20	15.6	35.7	13.6	18.3
N	11.4	11.6	*		16.9	48.1	16.5	44.7

*B.M.R. determinations were impossible during this period because the dog was too restless.

The average results of these experiments are shown in Table I.

Dogs W and N showed no appreciable difference in O_2 consumption during the period when the dogs received adrenal cortical extract with thyroid, and the following period, when the dogs received thyroid only. Dog B did show a difference, but during the period when thyroid and adrenal cortical extract were used simultaneously, the B.M.R. was higher than during the period when thyroid alone was used.

In view of our results on dogs and the reports of Oehme[5, 6] and Elmer, Giedosz, and Schepps[7] on guinea pigs, a more careful study is necessary, as to whether there is a species difference in the ability of adrenal cortical hormone to counteract the action of thyroid and thyrotropic hormone.

Summary. In dogs potent adrenal cortical extract does not prevent the rise in O_2 consumption induced by feeding thyroid.

I wish to thank Dr. Carlson for his advice during the course of these experiments.

10221 P

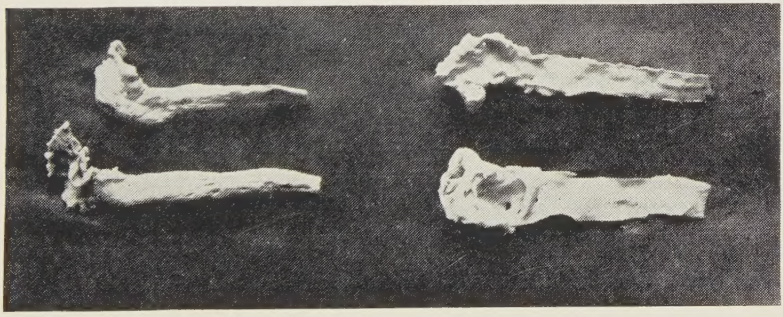
Action of Choline on Experimental Aortic Atherosclerosis.

ALFRED STEINER. (Introduced by Kenneth B. Turner.)

From the Research Division of Chronic Diseases, Department of Hospitals, City of New York, and the Department of Medicine, College of Physicians and Surgeons, Columbia University.

A previous communication[1] reported that the administration of choline to cholesterol-fed rabbits failed to prevent gross atherosclerosis, but did nevertheless result in a temporary delay in the appearance of aortic atheromata. The present study was designed to determine whether choline is effective in causing a reabsorption of the lesions previously produced in cholesterol-fed rabbits.

Twenty male chinchilla rabbits approximately 6 months old were used. The animals were kept indoors in individual cages. The diet consisted of oats, carrots and cabbage. One gram of cholesterol was mixed into the food of each rabbit 3 times a week for 110 days. The animals were then divided into 3 groups. Groups I-A and I-B, consisting of 5 animals each, were designated as controls. Group II contained 10 animals. Group I-A animals were sacrificed immediately after the 110-day cholesterol feeding period was completed. Group I-B animals were placed on the regular diet for 60 days after the cholesterol feeding period was discontinued. Group II animals were fed 0.5 g of choline daily for 60 days in addition to the regular diet after the 110 days of cholesterol feeding was completed. Groups



Group I-B (Control) Group II (Choline)

FIG. 1.

The 2 aortæ in group I-B are those from rabbits Nos. 310 and 406 which received cholesterol for 110 days and were fed a regular diet for an additional 60 days.

The two aortæ from group II are those from rabbits Nos. 244 and 245 which received similar feeding with cholesterol for 110 days and choline for an additional 60 days.

Note the absence of gross atheromata in aortæ of choline-fed animals.

1 Steiner, A., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 231.

TABLE I.
The Blood Cholesterol Values and Autopsy Findings in Groups I-A, I-B and II.

	Blood cholesterol mg/100 cc, days								Degree of Lipoid Infiltration			
No.	0	30	60	90	110	130	150	170	Aortic Atheromata	Liver	Adrenal	Kidney
Group I-A (Cholesterol for 110 days).												
248	156	500	500	454	454				+++	++	+	+
257	156	454	847	1204	1000				++++	++++	++	++
345	210	625	632	625	884				+++	++	+	+
249	147	333	561	436	456				++++	++	0	+
405	125	454	501	629	724				+++	++	+	0
Group I-B (Cholesterol for 110 days and regular diet for 60 days).												
109	153	586	724	700	357	370	394	284	+++	++++	++	+
265	146	442	833	100	1010	625	370	384	++	+	0	0
269	138	820	1081	1408	1234	666	649	500	++++	+++	++	++
310	147	416	746	662	625	714	606	500	++++	+++	+	+++
406	66	132	238	847	800	454		303	+++	+++	+	+
Group II (Cholesterol for 110 days, regular diet plus choline for 60 days).												
250	155	263	270	436	476	149	81	96	0	0	0	0
251	208	704	934	1041	1265	2000	1250	1250	+++	+++	+	+
255	132	276	372	490	512	357	108	125	0	0	0	+
244	131	282	282	413	500	123	208	104	0	0	0	0
335	144	285	568	892	900	746	624	544	++	+	+	+
344	118	316	355	526	592	431	275	500	+	+	++	+
379	126	303	352	350	400	263	200	240	0	0	+	0
381	127	666	655	800	1087	510	500	934	+++	+++	++	+++
382	114	314	400	434	385	361	140	125	0	0	0	+
327	130	213	417	333	454	582	666		0	0	+	0

I-B and II with one exception, were sacrificed on the 170th day of the experiment. One animal in Group II was sacrificed after 30 days of choline feeding on the 140th day of the experiment. The choline was administered by dissolving choline hydrochloride (Merck) in 5 cc of water and mixing with ground carrots. Food was withheld from the animals for 5 hours before the feeding of either cholesterol or choline to insure ingestion.

Blood was obtained from the ear vein at monthly intervals. Cholesterol determinations were made on the whole blood by the method of Bloor, Pelkan and Allen.[2] An autopsy was performed in each case. The extent of fatty change in the aorta, liver and kidney as shown macroscopically and the degree of adrenal enlargement was noted and graded from zero to 4 plus. The results are expressed in Table I.

From Table I it can be seen that 6 animals in Group II were entirely free of gross atherosclerosis, although the blood cholesterol in each was significantly elevated over a 4 to 5 month period. The question of spontaneous reabsorption of the aortic atheromata might be raised in view of the 60 day period of cholesterol-free diet previous to postmortem examination of the tissues. Anitschkow[3] and Scarff,[4] among others have shown that the characteristic arterial lesions do not disappear, but persist for periods up to 815 days following the withdrawal of cholesterol from the diet. This repeatedly corroborated finding together with the control data in the present series, would appear to deny the possibility of spontaneous reabsorption. It can also be seen in Table I that the 6 animals which failed to show aortic lesions exhibited no gross hepatic lipoid infiltration. This latter finding is consistent with the results obtained by Best and his coworkers[5, 6] in which choline was shown to relieve fatty livers in rats induced by feeding diets rich in fat or cholesterol.

The degree of lipoid infiltration in the kidney and of adrenal enlargement was similarly less in Group II.

Animal No. 327 was sacrificed after 30 days of choline feeding. It was of interest to note that lipoid involvement of the aorta, liver, and kidney was absent although the blood cholesterol level reached 666 mg/100 cc at the time of sacrifice.

From the evidence presented herewith, it is highly suggestive that choline causes reabsorption of the atheromatous lesions produced in the aortæ of rabbits by cholesterol feeding.

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3 Anitschkow, N., *Verhandl. d. deutsch. path. Gesellsch.*, 1928, **23**, 473.

4 Scarff, R. W., *J. Path. and Bact.*, 1927, **30**, 647.

5 Best, C. H., Ferguson, G. C., and Hershey, J. M., *J. Physiol.*, 1933, **79**, 94.

6 Best, C. H., and Ridout, H. H., *Ibid.*, 1936, **86**, 343.